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Studies on the Light-Reactions of Photosynthesis

in

Chlorella emersonii

by

FRANCIS WILLIAM MUSTO, B.Sc.

A Thesis submitted for the Degree of Ph.D.

University of Glasgow

Department of Botany

March 1983

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SUMMARY

The techniques of steady state and flash-induced oxygen evolution, flash-induced absorption spectroscopy and chlorophyll fluorescence spectroscopy have been used to study two aspects of the light reactions of photosynthesis in the unicellular green alga Chlorella emersonii.

(1) The effects of various compounds have been investigated in order to derive more information in elucidating the electrogenic mechanism responsible for the slow phase of the flash-induced absorption change at 515nm. Sub-uncoupling concentrations (circa 0.8 μ M) of carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) have been found to stimulate the apparent extent of the slow phase of Δ 515 and markedly decrease the rate of decay in Chlorella but not in chromatophores of Rhodospseudomonas capsulata or in intact or osmotically shocked chloroplasts isolated from Pea and Spinach. Similar results were observed using the other uncouplers carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) and 2,4 dinitrophenol (DNP), and the plastocyanin inhibitors, KCN and HgCl₂. Concentrations of the uncouplers CCCP, FCCP and DNP which induce these effects in Chlorella had no effect on steady state oxygen evolution or PSII photochemistry as measured by chlorophyll fluorescence spectroscopy. Photochemical blocking of PSII had no effect on the slow phase of Δ 515, but decreased the extent of the fast phase. The electrogenic step responsible for the slow phase is thus suggested to be associated only with PSI. Pre-illumination of Chlorella results in the disappearance of the slow phase; this effect may be reversed by dark adaption. Dark adaption takes place at an increased rate in the presence of 0.8 μ M CCCP, and therefore low concentrations of uncouplers may be seen to accelerate the dark process which brings about the slow phase of Δ 515.

Intact and shocked chloroplasts exhibiting the slow phase of $\Delta 515$ however require pre-illumination for the slow phase to be observed; these differences in light requirements for the slow phase to be present in the alga and chloroplast are accounted for by different redox poisoning requirements of the electron transport components responsible for the slow electrogenic step in the two photosynthetic systems. Concentrations of CCCP and KCN which stimulate the extent of the slow phase of $\Delta 515$ were found to decrease the rate of rereduction of cytochrome f but not to influence the redox kinetics of cytochrome b₅₆₃. The stimulation of the slow phase of $\Delta 515$ by the various compounds has been tentatively accounted for by two possible mechanisms. Firstly, by affecting the electrochemical potential across the thylakoid membrane, the compounds may influence the midpoint redox potentials of electron transport components associated with the extra electrogenic step and consequently correctly poise the components so as to allow the extra electrogenic step. Secondly, the slow phase of $\Delta 515$ may be present all the time but the decay under phosphorylating conditions is too fast for the slow phase to be seen. If phosphorylation is prevented by the various compounds and the decay of $\Delta 515$ slowed down, as has been observed, then the slow rise component will be more easily observed. The absence of the stimulation of the extent of the slow phase and decrease in the rate of decay of $\Delta 515$ in the chromatophores and chloroplast preparations is suggested to be due to the differing environments surrounding the thylakoids in the various in vitro preparations or to the loss of the components necessary for stimulation. This could be the result of the loss of loosely bound or stromal components or possibly an increased ionic permeability of the photosynthetic membranes brought about by the various isolation procedures.

(2) The effects of CuSO_4 , HgCl_2 , ZnSO_4 and $\text{Pb}(\text{NO}_3)_2$ have been investigated in order further to characterise the modes of toxicity of these heavy metal ions on photosynthesis.

Cu^{2+} has been found to; inhibit steady state oxygen evolution at approximately 10^{-4}M , quench the initial F_0 level, the maximum F_m level and the ratio $(F_m - F_0)/F_m$ of prompt chlorophyll fluorescence, as well as the Kautsky fluorescence induction. Furthermore a similar effect to that obtained with low concentrations of CCCP etc. was observed on $\Delta 515$, namely to stimulate the extent of the slow phase and decrease the rate of decay but also to decrease the extent of the fast phase. These results have suggested that Cu^{2+} acts firstly as an external quencher of exciton energy in the PSII, and possibly PSI, light harvesting pigments, and secondly Cu^{2+} may be seen to act in a manner akin to low uncoupler concentrations by affecting the ionic permeability of the thylakoid membrane either at the site of the coupling factor or in a non-specific manner over the whole thylakoid membrane.

Hg^{2+} has been found to cause similar effects to Cu^{2+} but with the additional factors; firstly of not quenching the F_v/F_m value, and secondly of markedly reducing the extent of the damping of the flash-induced oxygen evolution pattern. The results suggest that Hg^{2+} , like Cu^{2+} , acts as an external quencher of exciton energy in the PSII and PSI pigments and also behaves in a manner akin to low uncoupler concentrations. However, the above additional effects of Hg^{2+} suggest that it also acts at the water splitting complex site as well as possibly influencing the radiationless deactivation of exciton energy at the reaction centre of PSII.

Zn^{2+} was not found to inhibit photosynthesis markedly in these studies.

Pb^{2+} inhibited steady state oxygen evolution but this inhibition was found to be dependent on the presence of NaHCO_3^- and it is inferred that Pb^{2+} inhibited photosynthesis by removing the carbon source of photosynthesis.

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
B (R)	secondary electron acceptor of PSII
CCCP	carbonyl cyanide-m-chlorophenylhydrazone
CF	coupling factor
chl	chlorophyll
cyt	cytochrome
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone
DCCD	N,N'-dicyclohexylcarbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
DNB	m-2,4-dinitrobenzene
DNP	2,4-dinitrophenol
E	electric field strength
EDTA	ethylenediaminetetra-acetic acid
EPR	electron paramagnetic resonance
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Fd	ferredoxin
FeS	Rieske iron sulphur centre
F _m	maximum level of chlorophyll fluorescence
FNR	ferredoxin NADP reductase
F _o	initial level of chlorophyll fluorescence
FRS	ferredoxin reducing substance
F _v	variable prompt chlorophyll fluorescence (F _m - F _o)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HQNO	2-n-heptyl-4-hydroxyquinoline-N-oxide
HP	high potential
hν	photon of light
I	intensity of light
K _a	acid dissociation constant for a buffer
KCN	potassium cyanide
k _d	rate constant for radiationless deexcitation at the reaction centre
k _f	rate constant for fluorescence
k _h	rate constant for radiationless deexcitation

K_m	metal-buffer binding constant
k_t	rate constant for energy transfer from PSII to PSI
k_p	rate constant for photochemistry
k_q	rate constant for energy quenching
LP	low potential
M	molar concentration
MES	2-(N-morpholino)ethanesulphonic acid
MOPS	morpholinopropane-sulphonic acid
MEV	methyl viologen
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP
NEM	N-ethylmaleimide
$n(P^+)$	number of photochemical centres
$n(E)$	number of water splitting complexes
O-I-D-P-S-M-T	transient stages of the prompt chlorophyll fluorescence induction
P ₆₈₀	reaction centre chlorophyll of PSII
P ₇₀₀	reaction centre chlorophyll of PSI
PC	plastocyanin
P _i	inorganic phosphate
pmf	proton motive force
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
psu	photosynthetic unit
Q	first stable electron acceptor of PSII and quencher of fluorescence in its reduced form
RC	reaction centre
<u>Rps</u>	<u>Rhodopseudomonas</u>
Sn ($0 \leq n \leq 4$)	oxidation states of the oxygen evolving, water splitting complex
$t_{\frac{1}{2}}$	half time
TES	N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid
Tricine	N-tris(hydroxymethyl)methylglycine
TRIS	tris(hydroxymethyl)aminomethane
U,V	hypothetical reduced components necessary for the slow phase of $\Delta 515$
Y	primary electron donor to PSII
Z	secondary electron donor to PSII

$\Delta I/I$	change in absorption
Δ_{515}	flash-induced absorption change at 515nm
$\Delta \psi$	transmembrane electrical potential or gradient
ΔpH	transmembrane proton gradient
ϕ_F	quantum yield of fluorescence
ϕ_{pmax}	quantum yield of photochemistry

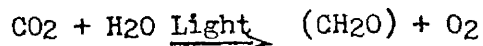
CHAPTER 1 : INTRODUCTION

1.1 General Introduction

After the conversion of hydrogen to helium by the fusion reaction in the sun, one of the most important reactions to life must be the ability of all autotrophic organisms to capture energy from sunlight and convert it to a form suitable for sustaining their own life and that of the rest of the living world. Photosynthesis as the process is termed not only provides the basic food source of all heterotrophic organisms but also maintains the atmospheric reservoir of oxygen. In addition its former activity has established the deposits of fossil fuels upon which man relies so extensively today. However, such resources are not endless and it has become increasingly apparent that alternative energy sources must be sought to sustain our expanding populations. An understanding of the molecular mechanisms of photosynthesis is therefore of utmost importance if we are to devise efficient artificial solar energy harvesting systems and to maximise the efficiency of those agricultural practices that we already rely upon. Finally it should not be forgotten that a process of such humanitarian importance is of great interest in itself.

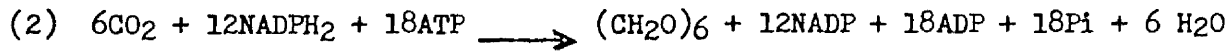
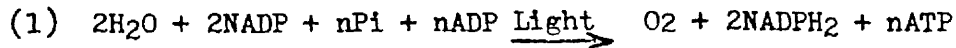
1.1.1 The Overall Process

The overall reaction carried out by green plants may be summarised as follows:



The overall reaction may then be divided into the processes occurring in the light and those occurring in the dark. In the light solar radiation is absorbed by photosynthetic pigments and the energy harnessed to: (1) oxidise a hydrogen donor resulting in the production of strong reductant, NADPH and (2) produce the "high-energy" intermediate, ATP.

In the dark the reductant NADPH and energy source ATP are used in the fixation of CO₂ to produce carbohydrates. The light and dark reactions may be summarised as follows:

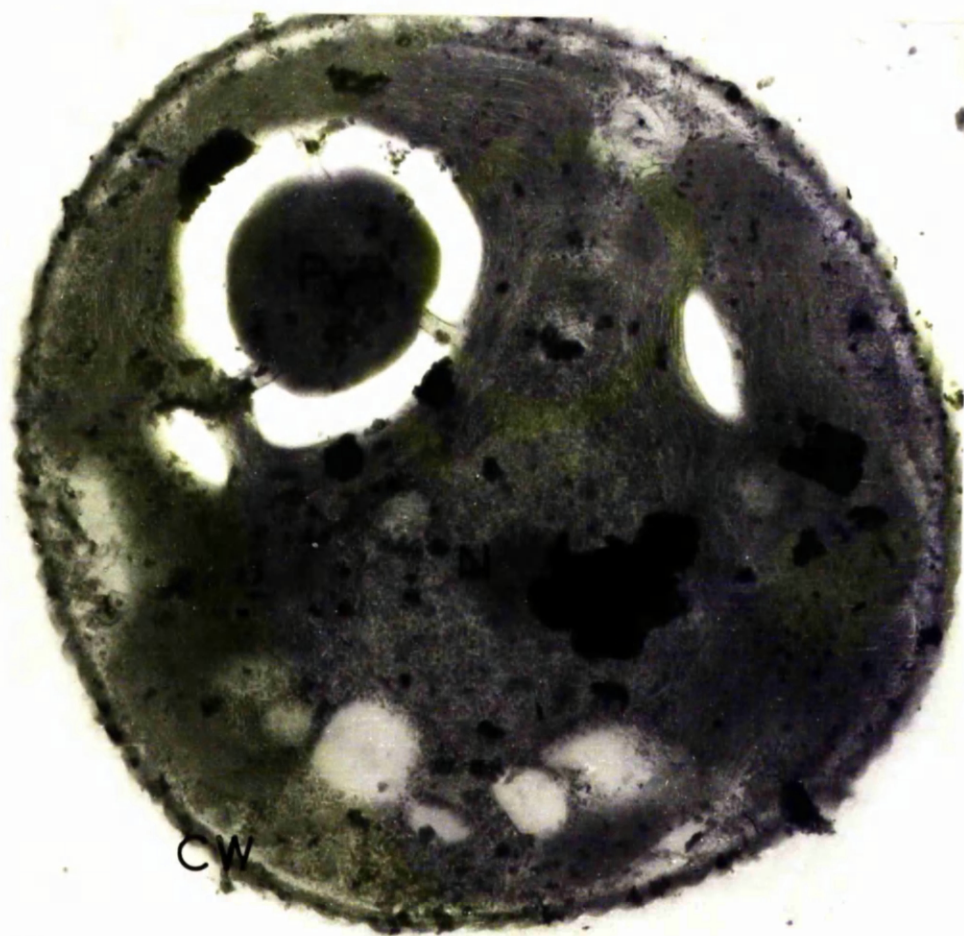
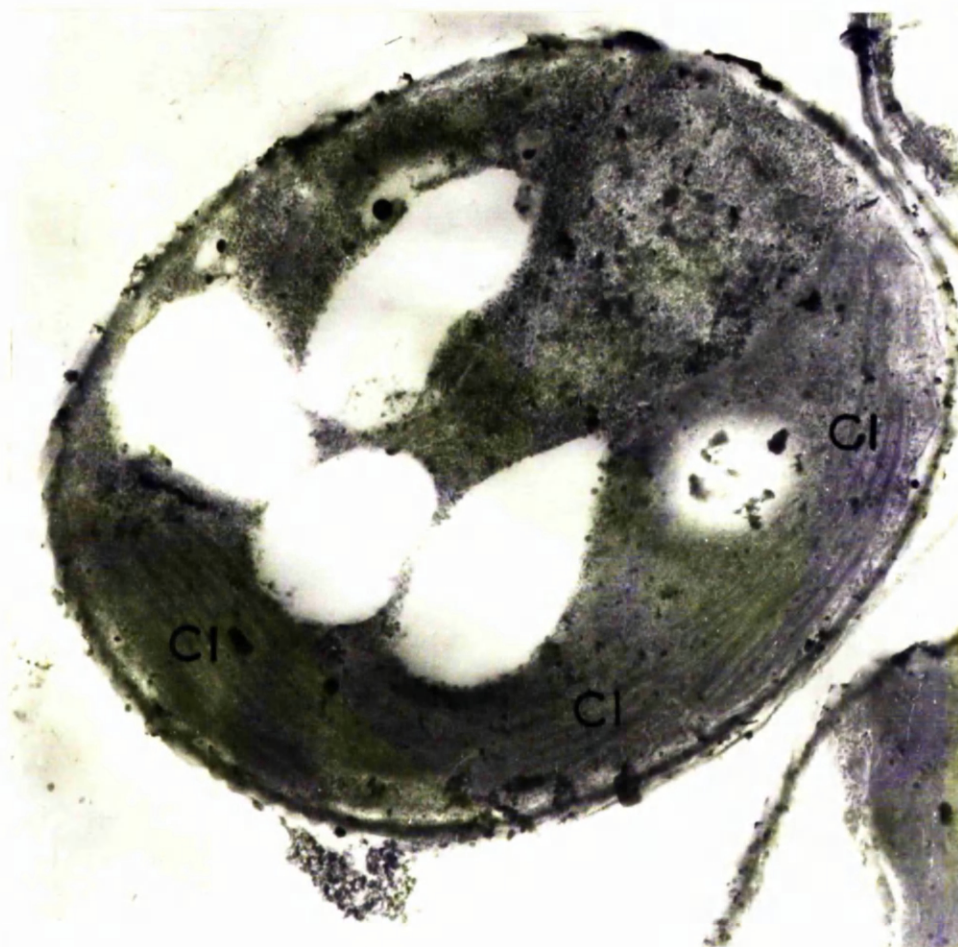


In algae and higher plants photosynthesis occurs in subcellular organelles called chloroplasts. The light reactions occur in and on the thylakoid membranes whereas the dark reactions take place in the stromal matrix. Higher plant cells contain many chloroplasts whereas unicellular algae such as Chlorella only contain a single chloroplast. (See Figure 1).

1.2 The Primary Processes of Photosynthesis

The process of photosynthesis commences with the absorption of a photon of light by pigments which are situated in the thylakoid membrane. The most important pigments are the chlorophylls, found in all the photosynthetic organisms, particularly chlorophyll a, the major chlorophyll found in all oxygen-evolving organisms, and bacteriochlorophyll the major one in bacteria. In addition, there are the accessory pigments of which the important ones in algae and higher plants are chlorophyll b and the carotenoids. The chlorophylls are non-covalently bound to specific proteins in pigment-protein complexes (see Thornber, 1975) causing several different absorption maxima in the red region of 650-700nm. The accessory pigments are also bound in such complexes and absorb in the blue-green region from 400-550nm. Research by Emerson and Arnold (1932) led to the concept that in higher plants and algae these pigments are organised into two functional "photosynthetic units"; photosystem I (PSI) and photosystem II (PSII) (see Figure 2).

Figure 1. Electron micrographs of Chlorella emersonii showing the single cup-shaped chloroplast (Cl), containing the thylakoid membranes, the pyrenoid (Py), the cell wall (CW), and the nucleus (N). Magnification x 24750.



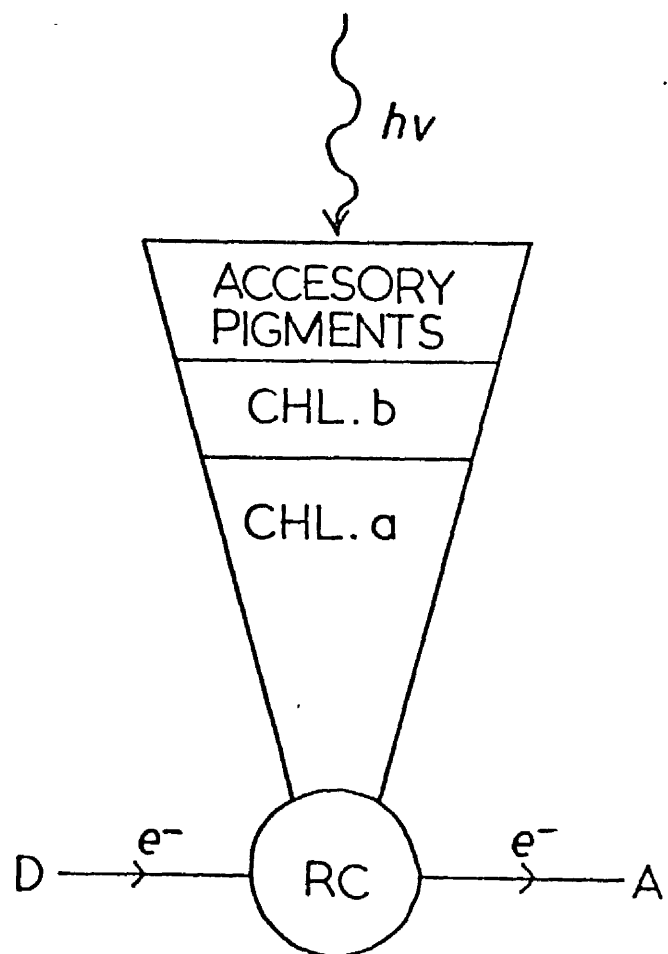


Figure 2. A schematic representation of the functional photosynthetic unit incorporating the light-harvesting pigments and the reaction centre. D is the primary electron donor and A is the primary electron acceptor.

Each functional unit is thought to comprise two types of pigment-protein complex; the majority of the pigment being found as a light harvesting pigment protein complex known as the antenna complex and a specialised longer wavelength absorbing chlorophyll a protein complex known as the reaction centre (RC) where the primary photochemical reactions take place.

The experiments of Emerson and Arnold (1932) using Chlorella indicate that each photosynthetic unit involved in the absorption of a quantum of light comprises 300 chlorophylls coupled to a reaction centre. It is not yet clear whether photosynthetic units are physical or statistical entities.

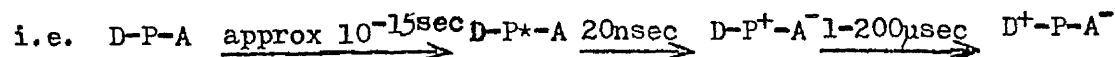
The absorption of light energy and its transfer to the reaction centre complex (see Sauer, 1975 ; Knox, 1977 for reviews) ~~may~~ be summarised as follows:

When a quantum of light is absorbed by a pigment molecule of the antenna complex an electron becomes excited and is promoted from a stable ground state to an unstable singlet state and the molecule is said to be in an "excited" state.

The potential energy of the molecule in the excited state may be dissipated in a number of ways. The molecule can return to its ground state through internal conversion with loss of the energy as heat in the kinetic motion of the molecule, or it can return to the ground state with the release of a quantum of light known as fluorescence (see section 1.5.2). Alternatively the absorbed energy can migrate from one antenna pigment molecule to another by the process of exciton migration so long as their singlet states correspond (Knox, 1977), until it encounters a reaction centre molecule.

In this way, it can be harnessed usefully by taking part in a chemical reaction at the reaction centre. This last possible fate for an exciton is the most important in photosynthesis but is by no means the only one that takes place (see section 1.5.2). Thus the purpose of the light harvesting pigments is effectively to increase the capture cross-section of the reaction centre.

The reaction centre consists of a specialised chlorophyll a molecule, (termed Chl a_{II} or P680 for PSII and Chl a_I or P700 for PSI) an electron acceptor A and an electron donor D. When a quantum of light energy reaches the reaction centre chlorophyll it becomes excited and donates an excited electron to the primary acceptor (Q for PSII and X or A for PSI). The oxidised chlorophyll molecule is then reduced more slowly by the primary donor. The chlorophyll molecule and the primary acceptor must both regain their original status before the process can be repeated. Thus the reaction centre complex or 'trap' may be termed 'open' or 'closed'



This oxidation-reduction reaction leads to a separation of charge between the components and an electric field is established (see section 1.5.4).

1.3 Electron Transport in Chloroplasts

The photochemical donation of an electron at the reaction centre and the subsequent acceptance of another from a donor begins a series of electron transfer steps which lead ultimately to the reduction of NADP and the oxidation of water to oxygen.

Hill and Bendall (1960) accounted for the classic experiments on enhancement and red drop of Emerson and Lewis (1943) by proposing that the two photochemical reactions were linked by a chain of intermediate electron carriers leading from water to NADP in the "Z Scheme" (see Figure 3). Much evidence has been accumulated in support of their original hypothesis and the Z Scheme is widely accepted (see reviews by Trebst, 1974; Avron, 1975; Golbeck et al. 1977 and Velthuys, 1980a). The passage of electrons from H₂O to NADP through the two photosystems is known as linear or non-cyclic electron flow. In addition to the production of NADPH the passage of electrons is also coupled to the formation of ATP from ADP and Pi (see section 1.4).

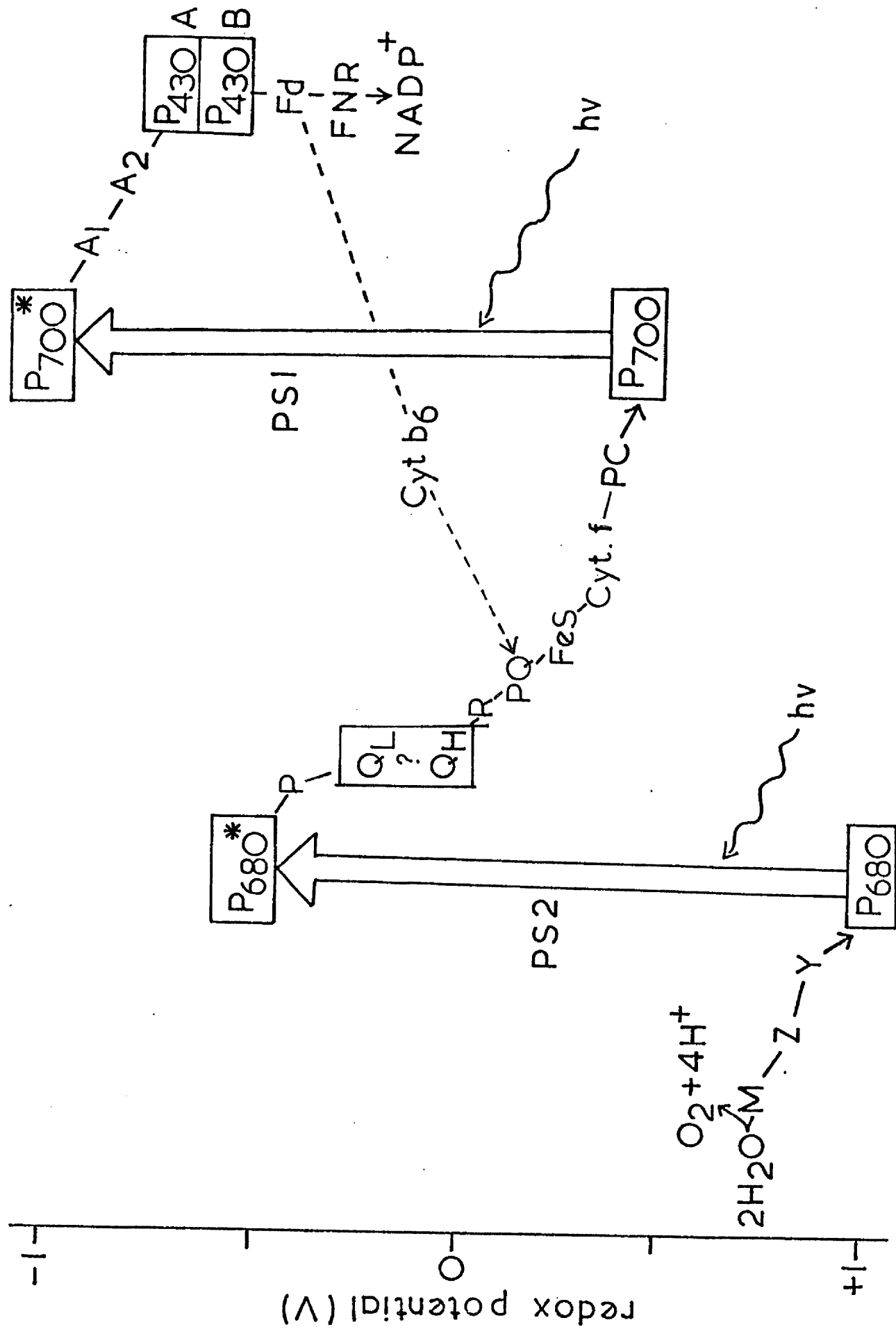
Coupled electron flow has also been reported around PSI with no overall oxidation of water and reduction of NADP (Whatley et al. 1956) (see review by Gimmeler, 1977). It is now established that "cyclic" electron flow plays an important role in vivo, providing ATP for CO₂ fixation and a number of other energy requiring processes in the chloroplast and cell (see Raven, 1976). The cyclic electron chain is thought to include a segment of the non cyclic pathway but includes an additional component, cytochrome b₅₆₃. (See Figure 3).

The various components of the linear and cyclic electron transport chains are summarised as follows.

1.3.1 The Donor Side of PSII (Govindjee, 1980 and Velthuys, 1980a)

This is one of the least understood parts of the electron transport chain. It comprises the water splitting complex and at least two electron carriers, one of which probably acts as the primary donor of PSII.

Figure 3. The "Z Scheme" of photosynthetic electron transport. Electrons pass from H_2O via the water splitting complex (M) and the two electron donors (Z) and (Y) to the reaction centre of PSII (P_{680}). From P_{680} the electrons pass to the PSII acceptors (P), possibly a pheophytin, $Q(L)$ and $Q(H)$ the first stable acceptor, and the secondary acceptor (R) which is thought to be a two electron carrier. The electrons are then passed to the reaction centre of PSI (P_{700}) via the intersystem carriers plastoquinone (PQ), the Rieske iron-sulphur centre (FeS), cytochrome f (Cyt f), and plastocyanin (PC). From P_{700} the electrons are passed to $NADP^+$ via the PSI acceptors (A_1), (A_2), (A, B or P_{430}) and ferredoxin (Fd) and the ferredoxin-NADP-reductase enzyme (FNR). The possible route for cyclic electron flow, via cytochrome b_{563} (Cyt b_2), around PSI is included (adapted from Govindjee, 1980).



The water splitting complex is thought to be situated on the inside of the thylakoid (Sane, 1977). Its capacity to evolve oxygen requires bound manganese, possibly as a charge accumulating species in the enzyme (Govindjee, 1980) and chloride possibly to stabilise the Mn complex (Govindjee et al. 1978). In addition, bicarbonate is implicated in O₂ evolution although it is thought to act on the reducing side of PSII. (Govindjee and Khanna, 1978). Spectroscopic and Electron Paramagnetic Resonance (EPR) studies have indicated that two electron carriers termed Z and Y function between the water splitting complex and the PSII reaction centre (Velthuys, 1980a). Sauer and co-workers (Babcock et al. 1976) have identified an EPR signal with the kinetics of Z. The presence of Y is suggested from kinetic measurements on the absorbance change due to P680, chlorophyll fluorescence rise kinetics after short flashes of light (Govindjee, 1980) and from EPR studies by Blankenship et al. (1977).

Important information on the mechanism of water splitting has been provided by measuring small amounts of oxygen released after excitation of photosynthetic material with a flash train (see section 1.5.1.1).

A number of treatments inhibit the water splitting complex; in inhibited systems electron donation to a number of sites may be achieved (see Hauska, 1977).

1.3.2 The PSII Reaction Centre Complex

The PSII reaction centre is thought to comprise a chlorophyll a molecule in a specialised environment termed P680 or Chl a_{II}, a donor Y (already discussed) and a primary acceptor generally termed Q. Q may be either a pheophytin molecule, the EPR signal of which was recently detected by Klimov, Ke and co-workers (Klimov et al. 1980 a and b) or it may be a special plastoquinone molecule (Knaff and Malkin, 1978).

Furthermore determination of the mid-point redox potential of Q by (i) fluorescence quenching (Horton and Croze, 1979) (see section 1.5.2) and (ii) the flash-induced 515 absorbance change (Malkin, 1978) (see section 1.5.3.1) suggests that there may be two distinct pools of Q of different redox potentials, -45mV and -245mV (Horton and Croze, 1979). Whether the two Q pools act in series, or independently in a parallel scheme is still to be resolved.

A small absorbance change at 690nm has been attributed to the PSII reaction centre (Doring and Witt, 1972; Doring et al. 1969). But, generally speaking, the reaction centre chlorophyll of PSII, P680, has been difficult to characterise; firstly because its rate of reduction is extremely rapid (about 30nsec), secondly because fluorescence changes occur in the same spectral region and time scale as the light induced bleaching of P680. Furthermore $\text{P680}/\text{P680}^+$ is a very strongly oxidising redox couple and it is therefore difficult to find a suitable redox reagent that will interact with P680 without also causing destruction of antenna chlorophyll (Malkin, 1982). [However, some insight into the reactions of PSII has been gained by indirect methods through monitoring three spectroscopic parameters of the primary electron acceptor: (i) an absorbance change at 320nm (Stiehl and Witt, 1968; Van Gorkom, 1974), (ii) an absorbance change at 550nm (Knaff and Arnon, 1969; Knaff, 1975), (iii) quenching of chlorophyll fluorescence by the primary electron acceptor termed Q (Papageorgiou, 1975). Knaff and Malkin (1978) have reviewed the properties of the components to which these spectroscopic parameters are assigned and the reasons for their assignment as the primary electron acceptor of PSII. In the context of this thesis a detailed examination of only the third parameter, chlorophyll fluorescence, is appropriate (see section 1.5.2).

1.3.3 The Electron Transport Chain Between the Two Light Reactions

The light reactions of PSII and PSI are connected by a chain of intermediate redox carriers; plastoquinone, cytochrome f, the Rieske FeS centre and plastocyanin. In addition, cyclic electron flow from the reducing side of PSI to P₇₀₀ via these interpool carriers is associated with a further carrier cytochrome b₅₆₃.

This transfer of electrons to the primary donor of PSI is energetically 'downhill' and the energy released is available to be chemically trapped in the form of ATP (see section 1.4). A summary of the components thought to make up the intersystem pool is considered here.

1.3.3.1 Plastoquinone (PQ)

Plastoquinone appears to have at least three possible roles in the intersystem electron transport chain; firstly as the primary electron acceptor of PSII (so far termed Q), secondly as the secondary electron acceptor of PSII termed B or R, and thirdly as a large pool of five to ten molecules of PQ per PSII reaction centre, kinetically observed to occur between the two photosystems (Stiehl and Witt, 1968). It is thought that the PQ pool links several PSII chains with several PSI chains (Witt, 1971). Plastoquinone may act as a 2 electron gate by accepting two electrons from separate PSII primary acts to form 2 plastosemiquinones. The two semiquinone anions then take up 2 protons from the stroma to form plastohydroquinone, PQH₂. The hydroquinone being of a hydrophobic character can diffuse across the thylakoid to the inner surface where the protons are released into the intra-thylakoid space and the electrons to the following electron acceptor (Rich, 1981).

Electron transfer from the hydroquinone to the following electron carrier (20msec) is likely to be the rate limiting step for the whole electron transfer chain (Rich, 1981).

1.3.3.2 The Rieske Iron-Sulphur Centre

An iron-sulphur centre has recently been detected, usually in chloroplasts, by Malkin and Aparacio (1975) with properties markedly different from the Fe-S centres already found to be located in the electron accepting side of PSI. At present, its exact location in the electron transport chain is tentative. However, its redox potential, its sensitivity to DBMIB (Chain and Malkin, 1979) and its presence in a digitonin extracted complex with cytochromes f, b₅₆₃ and b₅₅₉ (Rich et al. 1980), suggest that it is located on the reducing side of PSII in close proximity to PQ.

1.3.3.3 Cytochrome f and Plastocyanin

Although these two proteins are well characterised chemically (see Cramer, 1977 and Katoh, 1977), their exact ordering in the chain has proved difficult as they have the same midpoint redox potential. There have been a number of conflicting reports on the kinetics of the light-induced redox changes of the two carriers (Haehnel, 1973, 1977; Bouges-Bocquet, 1977 a and b; Bouges-Bocquet and Delosme, 1978). Both are reduced by PSII light and oxidised by PSI. Under conditions where plastocyanin is blocked either by inhibitors such as KCN or HgCl₂ (Trebst, 1974), or with plastocyanin-less mutants (Gorman and Levine, 1966), cytochrome f photo-oxidation was prevented but not its photoreduction. Haehnel et al. (1980) seem to have resolved the question with a detailed analysis of the kinetics of P700 reduction after short flashes. Their results suggest that cytochrome f donates electrons to plastocyanin which is the primary donor to P700 (for review see Malkin, 1982).

1.3.3.4 Cytochrome b_{559}

Although this electron carrier has been extensively studied its function and location in the electron transport chain remain unclear (see Cramer, 1977 and Malkin, 1982 for reviews). It is placed here for convenience along with the other cytochromes. It exists in two forms; a high potential form (HP) with a redox potential of +350mV (Knaff and Arnon, 1971) and a low potential form (LP) of +80mV (Bendall et al. 1971). Cytochrome b_{559} HP is closely associated with PSII particles (Wessells et al. 1973), it is photo-oxidised by PSII at cryogenic temperatures and may play a role in water splitting (Horton and Cramer, 1975) or in cyclic flow around PSII (Heber et al. 1979). Cytochrome b_{559} HP may be converted to the low potential form by ageing, mild heating and chemical treatment. Cytochrome b_{559} LP is associated with PSI (Knaff and Malkin, 1973) and also with the cytochrome complex obtained by digitonin treatment (Rich et al. 1980).

1.3.3.5 Cytochrome b_{563}

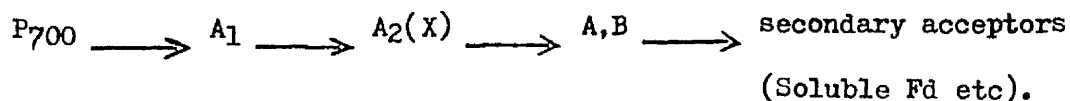
The function and location of cytochrome b_{563} remained unclear until recently when interest in cyclic electron flow around PSI has renewed interest in its behaviour. Its midpoint potential of -110mV has excluded its location in a simple linear sequence between the two photosystems. It is both photo oxidised and reduced by PSI light (Cramer and Whitmarsh, 1977). Furthermore antibodies to ferredoxin inhibit the reduction of cytochrome b_{563} (Bohme, 1977) and inhibition of plastoquinone by DBMIB prevents the photo-oxidation and reduction of cytochrome b_{563} (Crowther and Hind, 1980). Therefore cytochrome b_{563} has been assumed to act in a cyclic pathway round PSI, accepting electrons from ferredoxin and donating them to PQ. However, Bohme (1979) and Velthuys (1979) reported that the cytochrome can be photoreduced by PSII in a reaction which is sensitive to DCMU.

Furthermore the rate of electron flow from reduced ferredoxin to cytochrome b_{563} is slower than the light-induced reduction of the cytochrome (Olsen et al. 1980) and is too slow to support a significant turnover of cyclic electron flow (Telfer and Barber, 1981). It is therefore becoming evident that the behaviour of cytochrome b_{563} between the two photosystems is more complex than first thought. A more complex pathway must be involved and as this pathway is intimately involved with the slow phase of the electrochromic bandshift, it is discussed in more detail in that section (see section 1.5.3.5).

1.3.4 The PSI Reaction Centre

The reaction centre of PSI is composed of a specialised chlorophyll, P700, and an electron acceptor complex consisting of a number of chemically poorly characterised acceptors detected by EPR and optical techniques. P700 was first discovered by Kok (1961) as a reversible light-induced decrease in absorption (bleaching) at 700nm which he suggested to be the photochemical "trap" of PSI. Various evidence has been obtained since then which indicates that Kok's hypothesis was correct: Firstly, the oxidation of the specialised chlorophyll molecule occurs in a very short time (20nsec) (Wolff et al. 1969). Secondly, the light-induced oxidation takes place at cryogenic temperatures whereas the reduction is inhibited at such temperatures. Thirdly, the absorption difference spectrum of this irreversible change at cryogenic temperatures corresponds to the light minus dark difference spectrum of PSI (Witt, 1975). Furthermore, if ferricyanide is added to a chloroplast suspension in the dark the ^{light-induced} absorption change at 700nm is strongly decreased showing that ferricyanide oxidises P700 in the dark. This shows that P700 is oxidised in the light (Witt, 1971). No primary acceptor of PSI has yet been isolated, but its presence is inferred from EPR and optical observations of the light-induced reversible oxidation of P700 when all known acceptors have been blocked.

However, two iron-sulphur centres, A and B have been detected by the EPR technique at cryogenic temperatures and their redox potentials suggest them to act as rapid acceptors of PSI after the primary acceptor A₁ as follows (for review on PSI acceptors see Malkin, 1982).



A and B were also detected earlier by an absorption change at 430nm (Ke, 1973) and were thus known collectively as P₄₃₀. A further iron-sulphur centre A₂(X), detected by EPR, has been suggested to act before A and B (Golbeck et al. 1978) but the lack of sufficiently negative redox mediators has prevented its further characterisation.

1.3.5 The Reducing Side of PSI

Three reactions are thought to occur in the transfer of electrons from the primary electron acceptor of PSI to NADP: (i) the reduction of an iron-sulphur protein, ferredoxin (Fd) with electrons from the membrane-bound primary acceptor complex of PSI, (ii) a transfer of electrons in the dark from reduced Fd to the flavo-protein enzyme, ferredoxin-NADP-reductase; and (iii) transfer of electrons in the dark from the reductase to NADP (Whatley et al. 1963).

Ferredoxin was originally crystallised as an iron-sulphur protein by Tagawa and Arnon (1962). It is reduced by illuminated chloroplasts and donates electrons;

- (i) to NADP in the non-cyclic electron transport chain
- (ii) back to P₇₀₀ via cytochrome b₅₆₃ and PQ in the cyclic chain
- (iii) in a number of in vivo reactions such as the reduction of nitrite, nitrate, sulphide and sulphate, glutamate synthesis and fatty acid desaturation and N₂ fixation (see Arnon, 1977; Hall and Rao, 1977).

Ferredoxin is soluble but loosely bound to the external surface of the thylakoid membrane possibly via the NADP reductase enzyme and is thus easily lost if the chloroplast envelope is ruptured during isolation. Its structure and the evidence for its position in the electron transport chain is reviewed by Hall and Rao (1977).

The final reduction of NADP by Fd is catalysed by the flavoprotein, ferredoxin-NADP-reductase. This enzyme is relatively strongly bound to the thylakoid but is gradually lost by washing or ageing of chloroplasts (Trebst, 1974). Its structure and properties are reviewed by Golbeck et al. (1977) and Forti (1977).

An additional carrier termed ferredoxin reducing substance (FRS) has been reported by some authors to be necessary for the reduction of Fd. However, no redox properties of such a compound have been identified and it is now suggested possibly to play a structural role.

1.4 Photophosphorylation

Not only does the Calvin cycle require NADPH but also an energy source, ATP (see section 1.1.1). Photophosphorylation was first demonstrated in spinach chloroplasts by Arnon et al. (1954). A similar process of light driven phosphorylation was indicated in photosynthetic bacteria by Frenkel (1954). Three main hypotheses have been proposed for the mechanism of phosphorylation of ADP to ATP and the coupling of this process to light driven electron transport:

- (i) The chemical hypothesis (Slater, 1953) where electron transport leads to the formation of a covalently bonded "energy-rich" chemical intermediate. Splitting of this intermediate releases the energy necessary for the phosphorylation of ADP to ATP.

- (ii) The conformational hypothesis (Boyer, 1974) where electron transport generates an energy-rich conformational change in a protein. The energy is then passed from one protein to another via conformational transmission to the ATPase.
- (iii) The chemiosmotic hypothesis (Mitchell, 1961, 1966) (see Figure 4) where electron transport occurs vectorially across the photosynthetic membrane and is coupled to the pumping of protons into the intrathylakoid space. This results in an electrochemical gradient of protons being produced across the membrane. The production of ATP results from the efflux of protons through the coupling factor, ATPase, where the thermodynamic energy of the electrochemical potential difference is given up and is harnessed to phosphorylate ADP to ATP.

Over the last 20 years a great deal of research has led to the general acceptance of the chemiosmotic theory as the most likely mechanism of phosphorylation in chloroplasts, mitochondria and bacteria.

1.4.1 The Chemiosmotic Hypothesis Applied to the Chloroplast

Trebst (1974, 1980) has reviewed the evidence that electron transport in chloroplasts alternates from the inside to the outside of the thylakoid via a zig-zag pathway (see Figure 4). On absorption of light by the two reaction centre complexes, a vectorial transport of electrons occurs from the inner side of the membrane to the outside. This charge-separation between the primary electron acceptor and donor of the two photosystems results in a transmembrane electric potential difference being established in a time of less than 20nsec (Wolff et al. 1969).

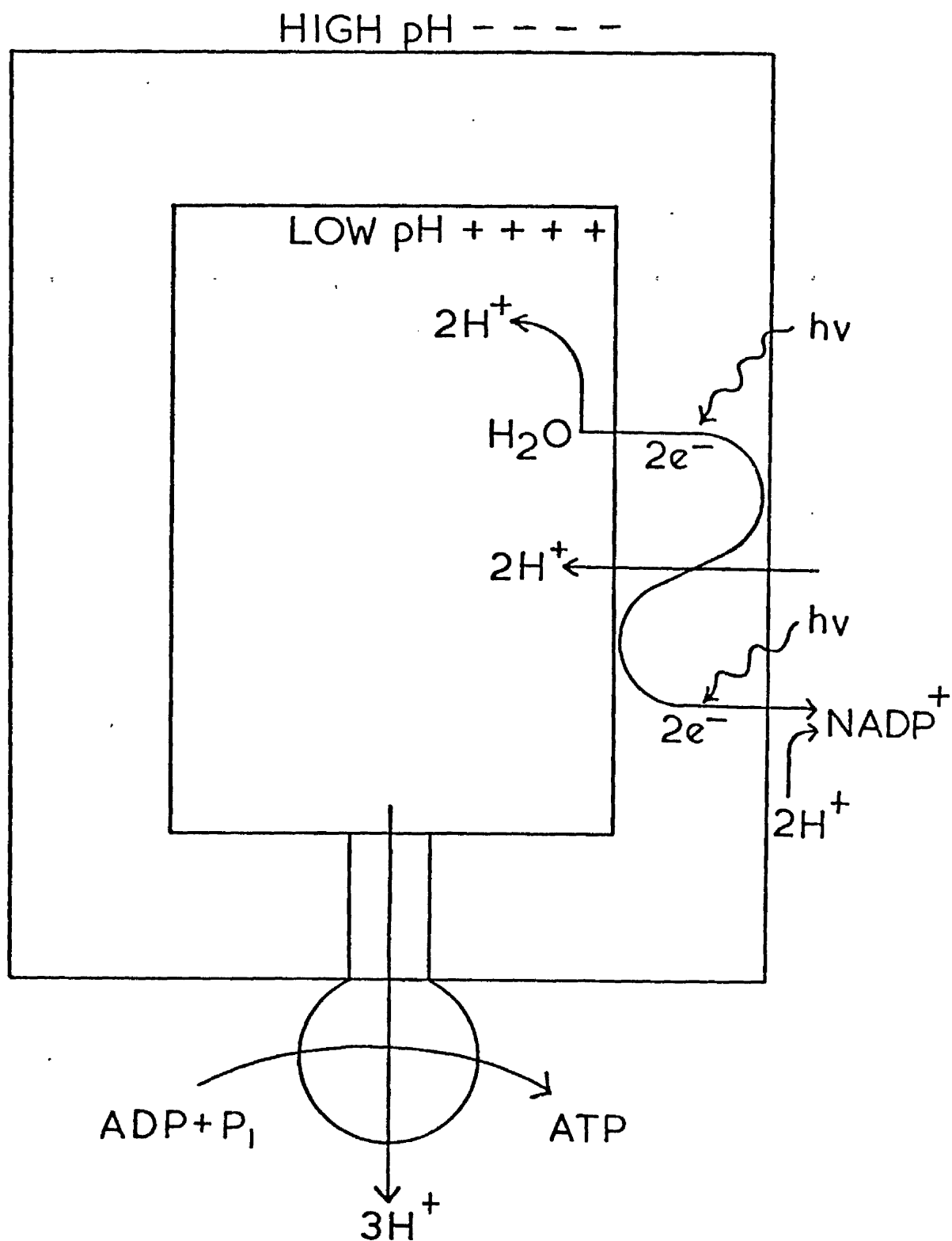


Figure 4. A schematic representation of photophosphorylation by the chemiosmotic method in the thylakoid. Light-driven electron transport is coupled to the inward pumping of protons resulting in an electrochemical gradient across the membrane. ATP synthesis occurs via the efflux of protons through the coupling factor (adapted from Hinkle and McCarty, 1978).

The resulting transfer of oxidising equivalents from PSII to the H₂O splitting complex leads to the oxidation of water and release of a proton inside the intrathylakoid space. As already described the electrons from PSII pass to PSI, via an intermediate pool of carriers. When the electron is transferred to PQ a proton is taken up from the stroma and translocated electro-neutrally to the inner surface where it is released. The electron then passes through the rest of the chain via PSI to NADP where a proton is taken up from the stroma and NADP is reduced.

Thus as the membrane is impermeable to protons there results an electrochemical gradient composed of two parts; a proton concentration term (pH) of 2 (Auslander and Junge, 1975) or possibly 3 (Velthuys, 1980b) protons per pair of electrons transferred and an electrical potential difference.

In the steady-state the excessive build up of electric charge is prevented by counter flow of other ions, particularly chloride influx and magnesium efflux (Hind et al. 1974; Barber et al. 1974 a and b) although some field remains.

The combination of the proton concentration difference and the electrical potential difference represent the eletrochemical high energy intermediate between photosynthetic electron transport and phosphorylation termed the proton motive force (pmf) where;

$$\frac{\text{pmf}}{(\text{mv})} = \Delta\psi + 2.303 \frac{RT}{F} \cdot \Delta \text{pH}$$

The final phosphorylation of ADP involves the efflux of protons through the CF₀ segment of the coupling factor, down their electrochemical gradient and the thermodynamic energy released is harnessed by the CF₁ catalytic segment of the ATPase to phosphorylate ADP to ATP (see Nelson, (1982) for review on the coupling factor).

1.4.2 Evidence for the Chemiosmotic Theory

Some of the more important pieces of evidence in favour of Mitchell's theory are summarised here. Firstly, Neumann and Jagendorf (1964) showed that an unbuffered solution of isolated chloroplasts takes up protons when illuminated. Secondly, phosphorylation has been induced by artificially producing

- (i) an electric field across the membrane by subjecting a chloroplast suspension to external voltage pulses (Witt et al. 1976).
- (ii) a proton gradient across the thylakoid membrane by equilibrating chloroplasts in a low pH medium and then transferring them to a higher pH (Jagendorf and Uribe, 1966).

Furthermore, the process is reversible and if excess ATP is present then protons may be pumped inward with the consequent hydrolysis of ATP to ADP (Mitchell, 1966). Thirdly, chemiosmosis should require a closed vesicle for the establishment of a pH gradient and chemicals which induce proton permeability in lipid membranes would be expected to inhibit phosphorylation. The "classical" uncouplers (CCCP, FCCP, DNP) (see appendix 2 for structural formulae) have the property of being weak lipophilic acids which can traverse membranes in their protonated form and return as an anion, thus making membranes proton permeable (Sybesma, 1977). This provides an additional pathway for the dissipation of the high-energy intermediate and does in fact prevent the proton-driven synthesis of ATP as expected from the chemiosmotic theory (De Kiewiet et al. 1965). The antibiotic, gramicidin, is not a proton-carrier but forms a transmembrane channel through which protons and other monovalent cations such as K^+ , Na^+ and NH_4^+ can pass. It too is a potent inhibitor of photophosphorylation (McCarty 1980). Other ionophores specifically dissipate the field component (valinomycin and K^+) or the pH component (nigericin or NH_4Cl) of the high energy state.

In order to prevent phosphorylation in chloroplasts both types of ionophore must be present indicating that photophosphorylation may be driven by either one of the high energy components (Izawa and Good, 1972).

Finally, the concept of an electric potential component of the high energy intermediate, postulated by Mitchell, has received a great deal of support from studies of an absorption change thought to reflect the electric field across photosynthetic membranes. This absorption change termed the 515 or 518 electrochromic shift has occupied a great deal of the research in this thesis and is dealt with in some detail in section 1.5.3.

1.5 Methods of Study of Photosynthesis

1.5.1 Oxygen Evolution

The source of electrons for photosynthetic electron transport in higher plants and algae is the oxidation of water, with the concomitant release of oxygen. Walker and Hill (1966) observed that changes in the rate of carbon fixation corresponded to changes in net oxygen production in isolated pea and spinach chloroplasts. Thus the rate of liberation of molecular oxygen gives a direct measure of photosynthetic electron transport. In intact chloroplasts and algae where the organelle contains the stromal components necessary for the dark reactions, the rate of oxygen evolution, after correction for respiration gives a measure of overall photosynthetic rate from H_2O to CO_2 fixation. In isolated chloroplasts which have been osmotically shocked the outer envelope is ruptured and the soluble stromal contents lost. Thus no terminal electron acceptor will be present and various artificial electron acceptors e.g. ferricyanide, methylviologen or NADP, must be added for oxygen evolution to occur. For the details on the measurement of steady state oxygen evolution see section 2.4.

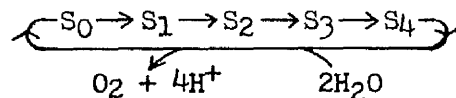
1.5.1.1 Oxygen Evolution at Photosystem II

The evolution of oxygen from water has the following stoichiometry:



Therefore four electrons must be removed from two molecules of water by PSII for a molecule of oxygen to be involved. PSII can only generate one positive oxidising equivalent per photoact (see section 1.2) therefore the storage of four positive oxidising equivalents produced by four photoacts must occur before oxygen is evolved.

Evidence for such a storage mechanism has been obtained from the measurement of oxygen evolution when Chlorella was illuminated by a series of short saturating flashes (Joliot et al. 1969). The relative amounts of oxygen yielded by each flash showed a damped oscillating pattern with a periodicity of four with no oxygen evolved on the first flash, a small amount on the second, and a maximum on the third (see Figure 5). Similar results were obtained by Kok et al. (1970) who provided a simple explanation. Assuming that each photoact was a one quantum process, equally efficient, and sensitized by the same pigment system and that each photoactive centre operated independently from other centres, Kok et al. (1970) proposed that the oxygen evolving system exists in five states S_0 , S_1 , S_2 , S_3 and S_4 with the subscript indicating the number of stored positive charges. As a consequence of each light reaction an S state is advanced to the next higher state until oxygen is evolved when S_4 reacts with H_2O as follows:



States S_1 and S_0 are relatively stable in the dark but S_3 and S_2 are not and decay to S_1 (Govindjee, 1980).

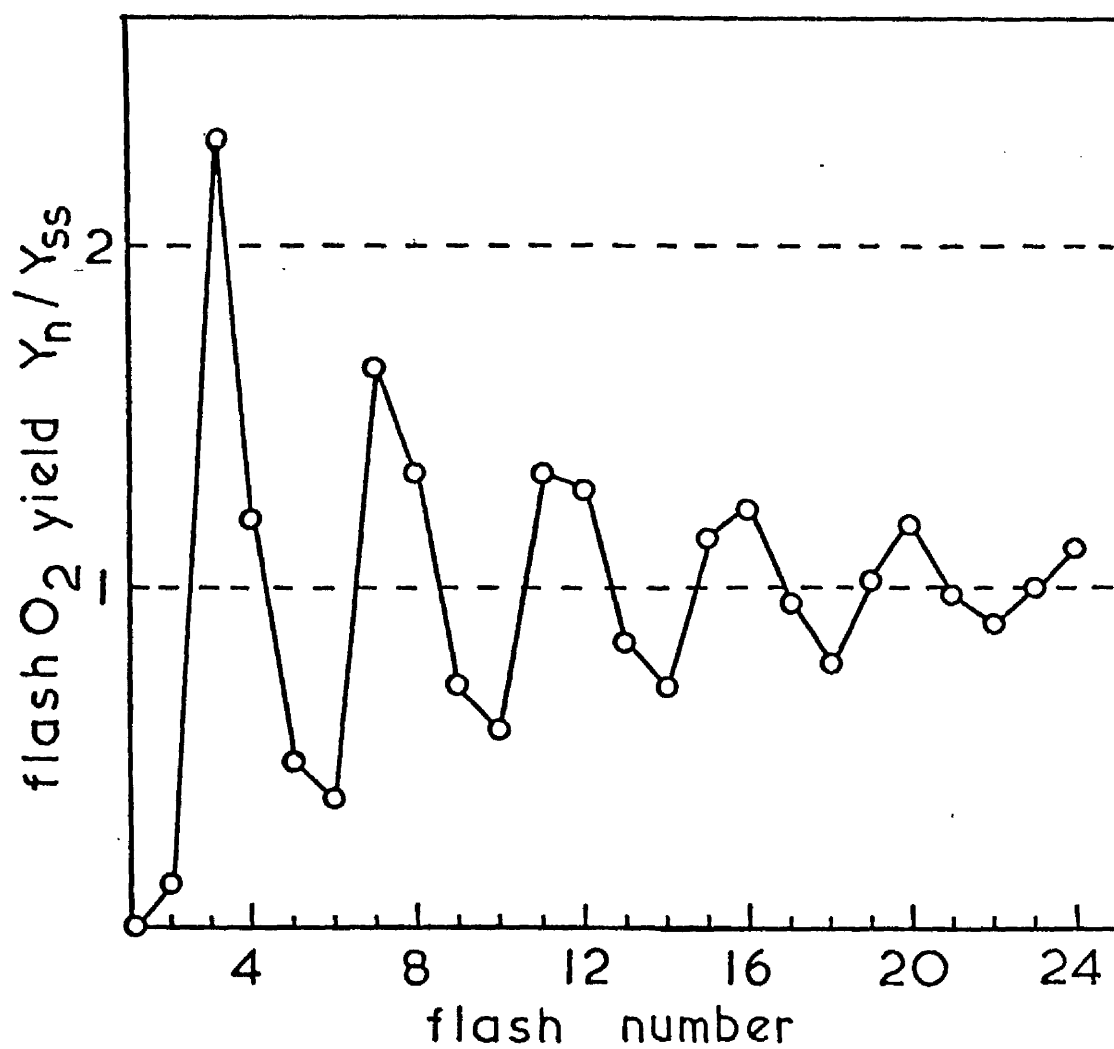


Figure 5. Flash-induced oxygen yield sequence observed with spinach chloroplasts after 40 minutes dark adaption (taken from Forbush et al. 1971).

In a solution of chlorophyll, however, about 30% of the absorbed quanta are re-emitted (Bowers and Porter, 1967; Govindjee and Govindjee, 1975), since the process which would normally use most of the energy, photosynthesis, is absent. At room temperature most in vivo fluorescence originates from chlorophyll a₆₇₈ in PSII with a maximum emission band at 685nm but PSI fluoresces very slightly at 740nm (Govindjee and Govindjee, 1975). At cryogenic temperatures fluorescence is emitted from chlorophylls of both systems with maxima at 684 and 695nm for PSII and 735nm for PSI (Govindjee and Govindjee, 1975).

Because prompt chlorophyll fluorescence varies inversely with the rate of photosynthesis (Butler, 1977) it has provided a useful probe for several phenomena, particularly the structure and function of the light harvesting pigments, the primary photochemical reactions and electron transport (see Butler, 1977; Lavorel and Etienne, 1977; Papageorgiou, 1975).

1.5.2.1 The Duysens and Sweers Hypothesis

The analysis of chlorophyll fluorescence in these fields has generally been interpreted using one major hypothesis, that of Duysens and Sweers (1963), as the starting point. They discovered that if the primary electron acceptor of PSII was reduced, either by PSII light, addition of dithionite or by blocking the re-oxidation of the acceptor with DCMU, then the fluorescence increased. When PSII was oxidised by PSI light then the fluorescence was quenched. Duysens and Sweers (1963) accounted for these observations by suggesting that at room temperature the fluorescence of PSII chlorophyll is governed by the redox state of the primary acceptor Q. When Q was not reduced, and the reaction centre, or "trap", open and able to receive an exciton and undergo photochemistry, the fluorescence was quenched.

When Q was reduced and the reaction centre unable to receive an exciton and undergo photochemistry the fluorescence was increased to a higher level. The theory may be quantified by reconsidering the fate of an absorbed quantum as follows. When a quantum of light is absorbed by a pigment molecule the trapped energy may follow one of several pathways:

- (i) radiationless internal conversion to heat via molecular vibrational, rotational or translational modes (h)
- (ii) transfer out of the array of PSII pigments to the weakly fluorescent pigments of PSI (t)
- (iii) trapping by a reaction centre molecule and driving the primary photochemical reaction (p)
- (iv) re-emission as a quantum of light i.e. fluorescence (f)

These processes may be assigned rate constants k_h , k_t , k_p and k_f respectively and the fraction of photons which follows each pathway, the quantum yields of each process, can be calculated as the fraction given by the rate constant of that process divided by the sum of all the rate constants. The quantum yield of fluorescence ϕ_f may thus be defined as

$$\phi_f = \frac{k_f}{k_f + k_h + k_t + k_p}$$

According to the Duysens and Sweers hypothesis (Duysens and Sweers, 1963), the rate constant k_p is further modified by the concentration of the available reaction centres capable of carrying out photochemistry. Hence, k_p is multiplied by the fraction of open traps, Q where $0 \ll Q \leq 1$. This leads to

$$\phi_f = \frac{k_f}{k_f + k_h + k_t + k_p \cdot Q}$$

Therefore as the traps shut during illumination Q will decrease and hence ϕ_f will increase.

1.5.2.2 Prompt Fluorescence (Induction) in the Presence of DCMU

If the Q to PQ electron pathway is blocked by an inhibitor such as DCMU then when dark adapted chloroplasts, with all their PSII traps open, are illuminated the time course of the closing of the traps may be followed as the fluorescence induction curve (Figure 6). On illumination the fluorescence immediately rises to the level, F_0 , where all the traps are open, and then rises more slowly to the F_{max} level where all the traps are shut (Malkin and Kok, 1966). The variable fluorescence between these two levels is attributed to the closing of traps and it follows that:

$$\phi_{F_0} = \frac{k_f}{k_f + k_h + k_t + k_p} \quad Q = 1$$

and

$$\phi_{F_m} = \frac{k_f}{k_f + k_h + k_t} \quad Q = 0$$

Using these easily measurable reference points it can be shown that (see appendix 3):-

$$\frac{\phi_{F_{max}} - \phi_{F_0}}{\phi_{F_{max}}} = \frac{k_p}{k_f + k_h + k_t + k_p} = \phi_{pmax} \quad (\text{Malkin and Siderer, 1974})$$

ϕ_p is the quantum yield of photochemistry of PSII and is thus an estimate of the efficiency of the photochemical reaction of that system.

However, maximum values of ϕ_p max which have been derived from the expression $(F_m - F_0)/F_m$ have been found to be consistently low (less than 0.8) with respect to values of greater than 0.95 obtained for the quantum yield of electron transport through PSII (see Butler, 1978). A number of recent reporters (Butler and Kitajima, 1975; Van Grondelle and Duysens, 1980; Haehnel et al. 1982) have accounted for this discrepancy between the measured and predicted F_m and F_0 levels by assuming that an extra radiationless deactivation pathway occurs in closed reaction centres.

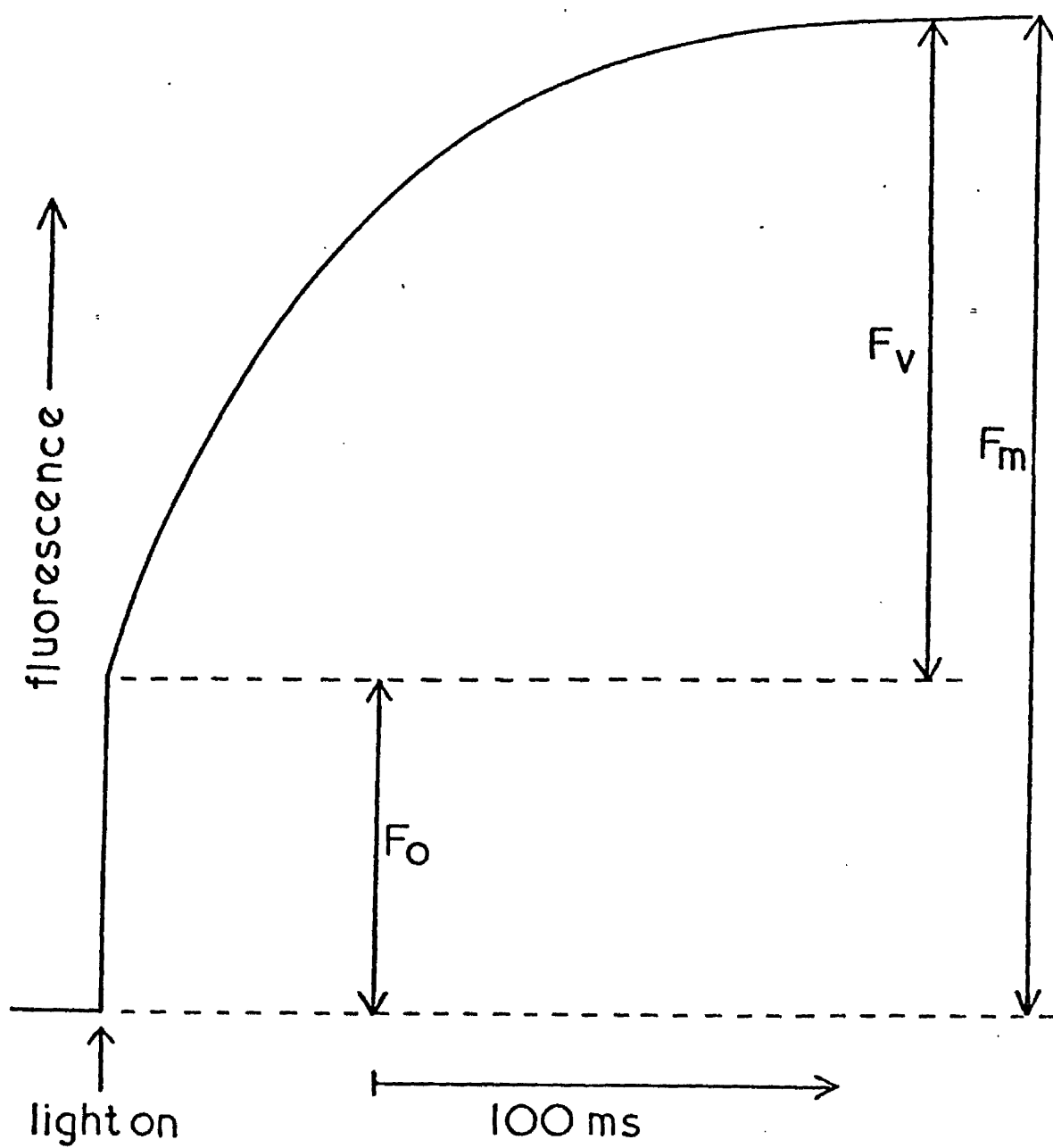


Figure 6. A typical fluorescence induction for Chlorella, dark-adapted for 5 minutes in the presence of DCMU.

The F_0 level will not be greatly affected since it is assumed that the rate constant for photochemistry, k_p , is much greater than the rate constant for radiationless deactivation at the reaction centre, k_d , and the other rate constants of deactivation k_f , k_h and k_t . However, when photochemistry is prevented by the reduction of Q, the radiationless quenching by the closed reaction centre becomes more significant with respect to the rate constants, k_h , k_t , and k_f . Thus closed reaction centres will show a marked quenching of the F_{max} level of fluorescence according to the rate constant, k_d . The expression $(F_m - F_0)/F_m$ will therefore only be equal to the quantum yield of photochemistry when the rate constant for quenching at the closed reaction centre is zero and at all other times should be modified as follows:

$$\frac{F_m - F_0}{F_m} = \phi_{pmax} \times \frac{k_p}{k_p + k_d}$$

1.5.2.3 The F_0 level of Fluorescence

Chlorophyll fluorescence quickly rises to the F_0 level on illumination of dark adapted material in less than 5µsec (Delosme, 1967). Thereafter, the F_0 level is assumed to remain more or less constant with time with variable fluorescence superimposed on it. The F_0 level is unaffected by the redox state of Q and is therefore insensitive to changes in electron transport. It is however sensitive to cation-induced structural changes (Hipkins, 1978) and also to agents, such as dinitrobenzene (DNB), which externally quench fluorescence by competing for the excitons in the light harvesting pigments (Etienne et al. 1974). The F_0 level is thought to arise from two possible sources. Firstly, the F_0 fluorescence may originate from pigments not related to PSII (Clayton, 1969).

Evidence for this theory was presented by Lavorel (1962) who showed the emission spectra of the F_0 and F_m levels differ particularly in the 720nm region and proposed that the PSI pigments may make a large contribution to the F_0 level. The second theory is that the non-variable fluorescence arises from de-excitation of PSII pigments before the exciton has been trapped by a reaction centre i.e. from competition between exciton transfer to open PSII traps and the alternative process of radiative decay (Delosme, 1967). This implies that the quantum yield of photochemistry is less than 100%.

1.5.2.4 Prompt Fluorescence in the Absence of DCMU

Fluorescence changes observed in intact photosynthetic systems in the absence of DCMU were first described by Kautsky (1931). The time-dependent kinetics are complex (see Papageorgiou, 1975 and Figure 7) and for convenience may be divided into a fast change of a few seconds and slower changes, termed O-I-D-P and S-M-T, respectively, by Lavorel (1959). [Essentially the fast changes reflect the extent of closed or open traps, as in the previous section, but with the qualification that as there is no block between Q and PQ, secondary electron flow can take place. Thus the fast fluorescence kinetics from O-P probably represent changes in electron transport rates through Q primarily from the water splitting complex to the pool of intersystem intermediates (see Papageorgiou, 1975; Lavorel and Etienne, 1977; Schreiber and Vidaver, 1976; Munday and Govindjee, 1969). The slower changes P-S-M-T, are not determined solely by the redox state of Q but are also intimately linked in with the induction of maximal rates of overall photosynthesis (see Walker, 1981).

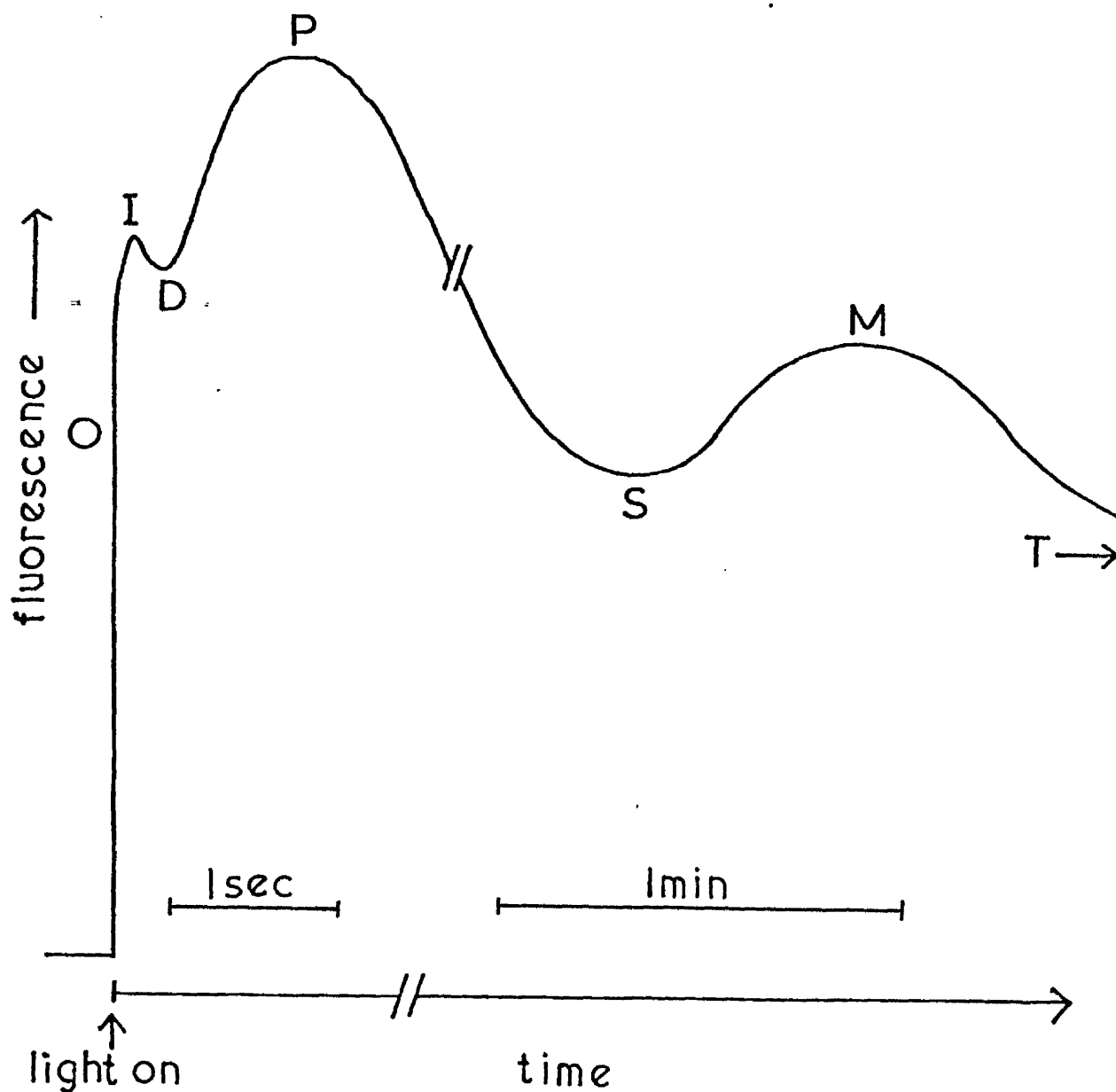


Figure 7. A typical fluorescence induction for Chlorella, dark-adapted for 5 minutes in the absence of DCMU. The symbols O, I, D, P, S, M, T are those originally used by Lavorel (1959) to describe the various transient phases of the curve.

The P-S-M-T stages may also reflect changes in the distribution of excitation energy between PSII and PSI, firstly in the initial distribution of incoming quanta to the two systems (Butler, 1977) and secondly by a change in the rate constant for energy transfer from the pigments of PSII to PSI (k_t), ("spillover") (Butler, 1977). This distribution of energy is thought to be controlled both by the stromal cation concentration (Barber, 1976) and by the phosphorylation of the light harvesting chlorophyll-protein complex by a plastoquinone-activated ATP-dependent protein kinase (Bennett, 1979).

The slow changes may also be partially caused by changes in the acidification of the intrathylakoid space and therefore by changes in the phosphorylation status of the system (Briantais et al., 1980).

Thus it can be seen that although there are still wide gaps in the interpretation of the various fluorescence parameters measured, it is an important tool in the study of photosynthesis particularly of the reactions of PSII.

1.5.3 Absorption Changes

Light-induced absorption changes in photosynthetic organisms have been attributed to three types of photosynthetic reaction; firstly the redox changes of electron transfer components, secondly the formation of metastable triplet states, and thirdly the electrochromic response of pigments to electric fields. Some examples of the first type of absorption change are those associated with the redox states of cytochrome f and cytochrome b₅₆₃, which may be observed by their absorption changes at 554nm and 564nm respectively (Dolan and Hind, 1974) and likewise the reaction centres of PSI and PSII at 700nm and 682nm (Witt, 1975).

A second type of absorption change, which occurs at 520nm, is attributed to the formation of metastable triplet states of carotenoids. The formation of these triplets provides protection for the light harvesting pigments from photo-oxidation (see Mathis, 1977 and Witt, 1971).

A much larger change was first reported by Duysens (1954) with a maximum at 515 - 520nm and minima at 478 and 420nm. This absorption change has since been the subject of a great deal of research and is thought to reflect a different type of mechanism.

1.5.3.1 The 515 Absorption Change (Δ 515)

The absorption change first observed by Duysens (1954) was investigated by Witt and Moraw (1959). The change had a fast rise time of less than 10^{-5} sec and decayed biphasically in about 20 msec depending on the physiological state of the membrane (Junge and Witt, 1968). It has been observed at different wavelengths in all photosynthetic systems; algae, higher plants and bacteria.

1.5.3.2 The Mechanism of Δ 515

Δ 515 is thought to reflect an electrochromic band shift of the photosynthetic pigments caused by an electrical potential across the thylakoid membrane (see section 1.4). Electrochromism is the effect of strong electric fields on the absorption spectra of dye molecules, due to an interaction of the excited + ground states with the electric field vector E

$$\Delta v = 1/h (\mu^* - \mu^0)E + 1/2 (\alpha^* - \alpha^0)E^2$$

Where $\Delta\nu$ is the frequency shift, μ is the dipole moment of the molecule, α its dielectric polarizability and the subscripts * and ° refer to excited and ground states respectively (Junge, 1977). These vectors μ and α have definite orientations within molecular co-ordinate systems and therefore the frequency shift will depend on the orientation of the dye molecule relative to the direction of the electric field. Molecules with a centre of symmetry such as the carotenoid pigments in the thylakoid membrane have no permanent dipole moment either in the ground or excited state and therefore the electrochromic shift of the photosynthetic pigments should depend by second order on the electric field strength according to the above equation (Junge, 1977).

1.5.3.3 Evidence for the Light-Induced Absorption Change at 515nm as an Electrochromic Response of the Photosynthetic Pigments

The evidence which has led to the acceptance of the absorption change at 515nm as an electrochromic response to the light-induced membrane potential may be divided into three main areas as follows:

(i) Kinetic Evidence

Figure 8 shows a typical time course of the light-induced absorption change at 515nm. The fast phase of increase has been attributed to primary charge separation caused by vectorial electron transport at the two reaction centre complexes for the following reasons. Firstly the onset of $\Delta 515$ occurs in a similar time range of less than 20 nsec (Wolff et al. 1969) to that of the primary reactions at the two centres. Secondly, by separately inhibiting each photosystem by optical or chemical means Schliephake et al. (1968) showed that the extent of $\Delta 515$ was halved in each case.

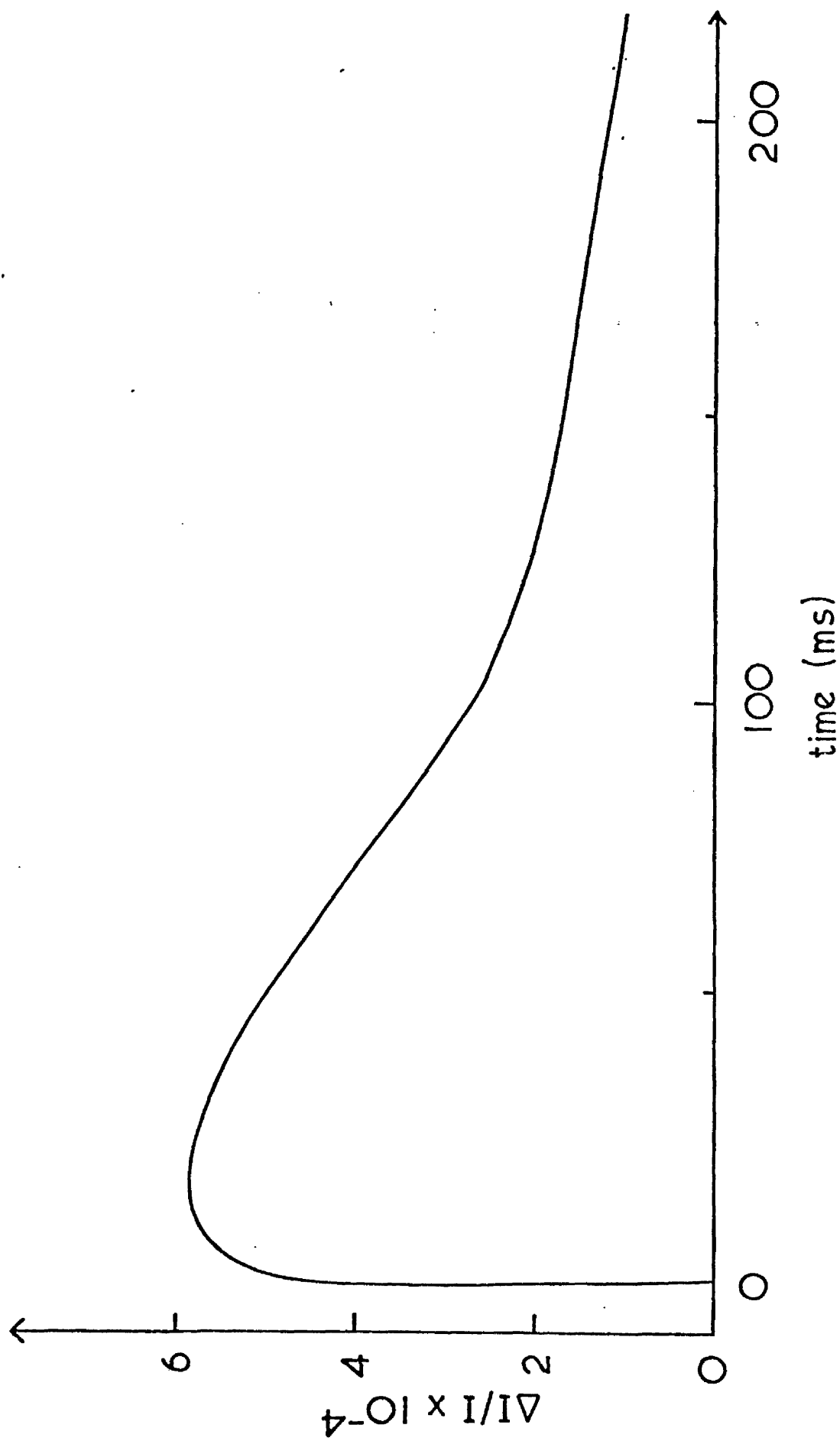


Figure 8. A typical time course of the flash-induced absorption change at 515nm in Chlorella, dark-adapted for 5 minutes (chlorophyll concentration circa 30 $\mu\text{g ml}^{-1}$).

The same group also showed that the action spectrum of $\Delta 515$ corresponded to the action spectrum of the two photosystems (Schliephake et al. 1968). A different line of kinetic evidence has come from studies of light-induced asymmetric electrostatic induction. The principle of electrostatic induction is that excitation of a chloroplast suspension with a non-saturating flash will produce more photosynthetic charge separation in the membrane of a thylakoid vesicle nearer the light. This results in the generation of net dipoles with their negative parts toward the light source. The potential generated can be detected by macro electrodes spatially arranged along the axis of light propagation (see Fowler and Kok, 1974 and Witt and Zickler, 1973). This provides an alternative, accurate method of studying initial light-induced electrical events in chloroplasts with a high time resolution. Using this method, Graber and Trissl (1981) observed the risetime of the photovoltage in a suspension of chloroplasts to be less than or equal to 4.8 nsec which was in agreement with the rise time of $\Delta 515$. Furthermore the photovoltage was halved when each of the photosystems was inhibited in a similar manner to $\Delta 515$ (Fowler and Kok, 1974).

Various studies on the effects of ionophores, and other treatments which effect the ionic permeability of the thylakoid membrane, on the rate of decay of $\Delta 515$ have indicated that the decay represents the decay of the transmembrane electrical potential by ionic flux across the membrane. Junge and Witt (1968) showed that the rate of decay may be accelerated by a number of treatments:

- (i) increasing the permeability of the membrane to alkali ions by the ionophore gramicidin;

- (ii) an increased concentration of alkali ions in the presence of gramicidin in the suspending medium of the chloroplasts;
- (iii) increasing the mobility of the alkali ions present with gramicidin i.e. Cs^+ and K^+ are greater than Ca^{2+} and Mg^{2+}
- (iv) osmotic stress of the membrane in hypo- and hyper-tonic bathing solutions;

Treatment of thylakoids with "classical" uncouplers, which make the membrane specifically permeable to protons (see section 1.4.2), such as DCPIP (Witt and Muller, 1959) and CCCP (Rumberg and Siggel, 1968) transformed the slower of the two kinetic phases of decay into the fast one whilst increasing the rate of electron transport. The alkali ion ionophores did not induce this. Secondly the rate of decay of the slow phase of decay has been linearly correlated with the phosphorylation activity (Rumberg and Siggel, 1968; Junge and Witt, 1968). Thus the decay of the absorption change is thought to represent the decay of the electrical potential difference across the thylakoid by ion flux, specifically by proton efflux under phosphorylating conditions.

(ii) Evidence from Artificially Induced Diffusion Potentials

Jackson and Crofts (1969) imposed artificially induced electrical potentials across the photosynthetic membranes of chromatophores of Rhodospseudomonas spheroides by means of ionic gradients operating through ionophorous antibiotics (e.g. valinomycin and K^+) and uncoupling agents. They found that a similar shift to the light-induced shift in the carotenoid spectrum was induced in the dark when a potential was generated which was positive with respect to the inside of the chromatophores. Generation of a negative potential with regard to the inside of the vesicle gave a mirror image of the spectral change induced by light. They further showed a linear relationship between the extent of the absorption change and the diffusion potential.

Similar results were obtained using chloroplasts by Strichartz and Chance (1972) who attempted to produce an electrical potential difference across the thylakoid membrane by addition of strong concentrations of alkali cation salt solutions. However, significant differences between the light-induced and salt-induced difference spectra suggested that the addition of cations was inducing absorbance changes other than electrochromic ones. These were probably cation-induced structural changes in the membranes causing changes in light scattering (see Gross and Libbey, 1972; Gross and Prasher, 1974; Telfer et al. 1976). Thus, Shapendonk and Vredenburg (1977) repeated the experiments in the presence of low concentrations of $MgCl_2$ and using split beam and double beam spectrophotometric methods to minimize scattering changes. The difference spectrum in the 460-540nm wavelength region they obtained upon addition of KCl in the presence of valinomycin was identical with the light-induced spectrum attributed to the electrochromic shift.

(iii) Spectroscopic Evidence

Spectroscopic evidence for the 515 absorption change has arisen from the similarity between the flash-induced difference spectrum in chloroplasts and the difference spectra obtained by applying a large electric field across chloroplast suspensions and artificial layers of chloroplast pigments. Schmidt et al. (1972) incorporated monolayers of the various pigments thought to be involved in the absorption change, chlorophyll a and b and various carotenoids, into a thin light-transmitting capacitor. The in vitro difference spectra they obtained for the different pigments, when superimposed, were similar to the in vivo difference spectrum obtained in chloroplasts (Emrich et al. 1969) with the exception of the region from 670nm onwards. The individual pigment spectra indicate that all three pigments are involved; the carotenoids being responsible for the positive peak at 515nm and chlorophyll b for the negative one at 478nm.

Stritchartz (1971) however showed that heptane extraction of β -Carotene from spinach chloroplasts dissipates the absorption change at 518nm and re-addition of β -Carotene partially restores it.

The response of the pigment monolayers showed a quadratic dependance on field strength which differs from the linear dependency observed in the in vivo systems of chloroplasts (Schliephake et al. 1968) and bacteria (Jackson and Crofts, 1969). This discrepancy has been accounted for by Schmidt et al. (1972) who suggested that the pigments in the photosynthetic membrane, responsible for the absorption change, are exposed to a relatively large permanent field resulting from polar and dipolar groups of adjacent molecules. If the light-induced field acts as a small addition to an already large field then the second order absorption response would approach a first order pseudo-linear response (see Figure 9) (Junge, 1977).

That a quadratic field dependence does occur in chloroplasts was shown by measuring the effects of external electric fields on the absorption spectra in osmotically swollen chloroplasts or "blebs" (Schloder and Witt, 1980 and De Grooth et al. 1980a).

They proposed that the difference spectrum of the "blebs" is not linearly dependent on the field strength, as found for the light-induced spectra in chloroplasts and bacteria (Schliephake et al. 1968 and Jackson and Crofts, 1969), because these linear dependent field changes are caused by pigments possessing a permanent dipole moment induced by a permanent field imposed by their local environment (see above). This field is directed from the inside of the membrane to the outside. The externally applied field however induces a field directed towards the inside for half of the vesicle and outwards for the other half.

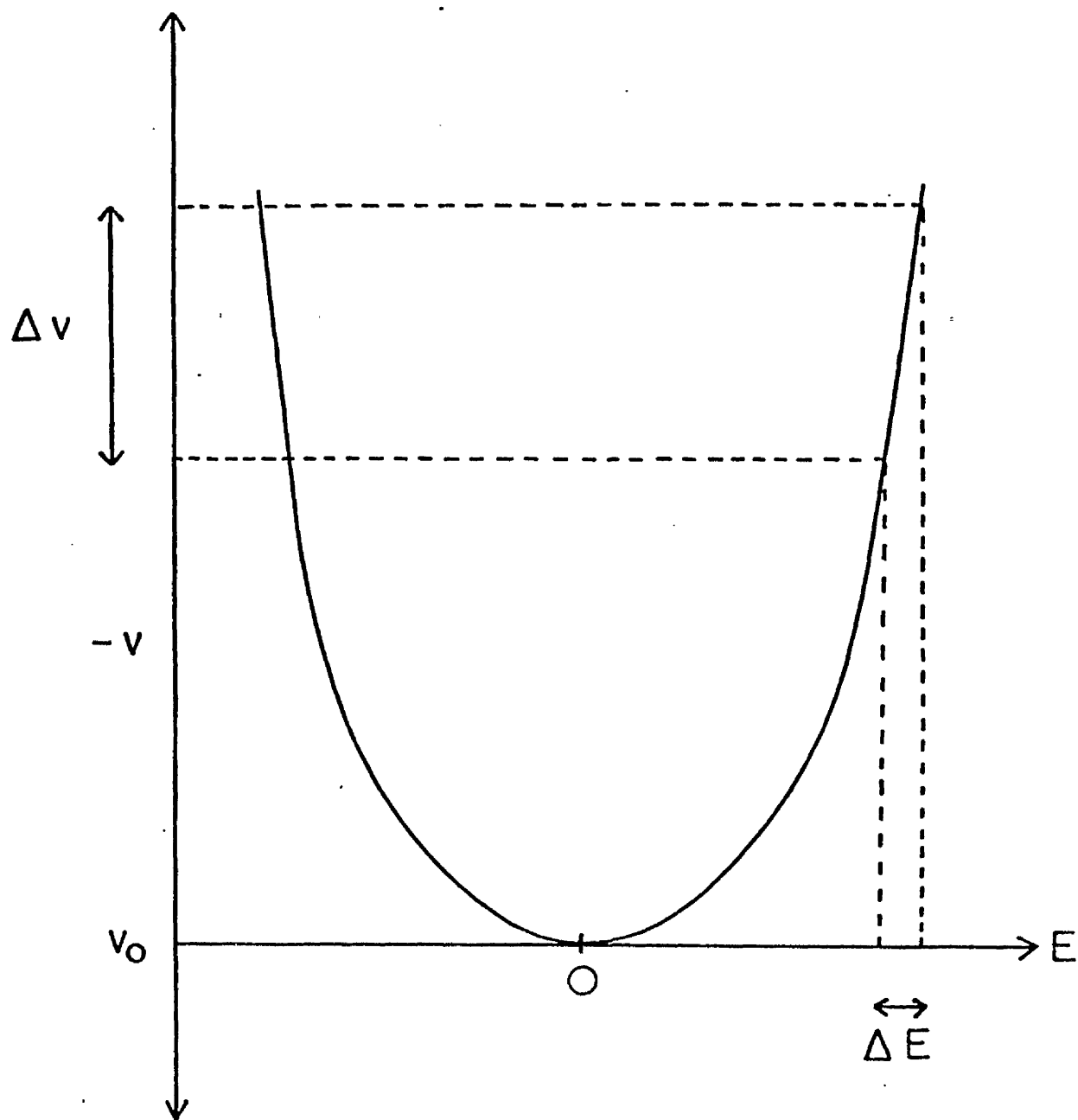


Figure 9. Possible explanation for the linear dependence of the absorption change at 515 nm on the field strength across the thylakoid membrane. If the pigment molecules are exposed to a large permanent electric field (E) the second order dependence of the frequency(v), and hence wavelength, of absorption of the pigments may become pseudo first order (Δv) with respect to the smaller variable light-induced field change(ΔE).

Thus absorption changes determined by the permanent field, which depend linearly on the field strength, will be cancelled because for each molecule which undergoes a red shift there will be a molecule located on the opposite side of the "bleb" which undergoes an equal blue shift. On the other hand the absorbance spectra of all carotenoids will be shifted towards longer wavelengths irrespective of their location in the "bleb" wall as a consequence of quadratic electrochromism.

A method of separating the absorption changes of each half of the "bleb" was shown by De Grooth et al. (1980b). They used a lens and diaphragm to focus only that part of the measuring beam passing through one half of the "bleb" onto the photo multiplier. Their results suggested that the linear dependence of the absorption change induced by an electric field was much larger than the quadratic one and more importantly the difference spectrum of linear electrochromism induced by an external field is indeed nearly identical to the light-induced, uncoupler-sensitive absorption change at 515nm.

These pieces of evidence have led to the acceptance of the original interpretation made by Junge and Witt (1968) and Jackson and Crofts (1969) that the absorbance change at 515nm in chloroplasts and 523nm in bacterial chromatophores is an electrochromic response of photosynthetic pigments (mainly carotenoids) to a light-induced electric field across the thylakoid membrane, proposed by Mitchell as part of the driving force for phosphorylation.

1.5.3.4 Limitations of the Electrochromic Absorption Change

Allowing for the discrepancy of the linear dependency of the absorption change on the electric field, as already discussed, the following limitations must also be considered when using the electrochromic absorption change to study the membrane potential and related electric phenomena.

As has been mentioned in conjunction with the salt pulse studies, structural changes in the thylakoid membrane cause changes in light scattering that are detected as apparent absorption changes at 515nm. Thorne et al. (1975) monitored the light scattered at 90° by illuminated intact chloroplasts in relation to the 515nm light-induced absorbance change. They observed a direct correlation between the light-induced scattering changes at 90° and the slower component (greater than 1 sec) of the electrochromic absorption change. They concluded that the slower component (greater than 1 sec) of the absorption change at 515nm was caused by an increase in light scattering due to conformational changes of the thylakoid membrane, not electrochromism. Garab et al. (1979) continued these studies in the shorter time range from 10 μ sec - 5 secs in Chlorella. They showed that the kinetics of the flash-induced absorption change and those of the flash-induced scattering transients, measured at 90° relative to the measuring beam, were identical within the first 500 msec but diverged considerably after 500 msec. They showed that fast transients in scattering and absorption are physically interrelated i.e. fast scattering changes (less than 500 msec) can also be interpreted as an electrochromic phenomenon. The slower changes of both signals were accounted for in a similar way to Thorne et al. (1975).

Their data show that absorption changes at 515 - 520nm may only be interpreted in an electrochromic manner within the first 500 msec following a flash.

A further absorption change which is superimposed on the electrochromic one at 515nm is that of the carotenoid triplet. This absorption change rises with the same time course (less than 20 nsec) as the electrochromic absorption change but decays in a considerably shorter time (half time of decay 3 - 7 μ secs, Joliot et al. 1977).

Although the difference spectrum of the triplet formation has a positive band very similar to that of the electrochromic effect at 510-520nm, the contribution of the metastable triplet state at 480nm is negligible (Joliot et al. 1977). Thus where the electrochromic absorption change is being studied in the time scale of 10-50 μ secs the contribution of the triplet state may be determined from the spectral differences at 515 and 480nm.

1.5.3.5 The Slow Phase of Absorption Increase at 515nm

In addition to the fast phase of increase in absorption at 520nm a slower increase in absorption with a half rise time of several milliseconds was first observed in algae (Witt and Moraw, 1959 and Joliot and Delosme, 1974) and more recently in intact chloroplasts (Horvath et al. 1979 and Slovacek and Hind, 1978). This slower second phase of increase has not been well investigated and it is only recently that attention has been focused on it. Early studies by Joliot and Delosme (1974) on Chlorella suggested that it was only associated with PSI and was dependent on the physiological conditions, such as temperature and light pretreatment under which the Chlorella were incubated at the time of measurement. Other than this however it was unclear as to what the slow phase of absorption increase represented.

1.5.3.5.1 The Mechanism of the Slow Phase of Δ 515

Three possible mechanisms might be put forward to interpret the slower phase of absorption increase. Firstly, it may be that the primary photochemical charge separation does not occur right across the membrane from the inner to the outer face only through a part of it. For complete charge separation from one side of the membrane to the other an additional charge separation step would be required.

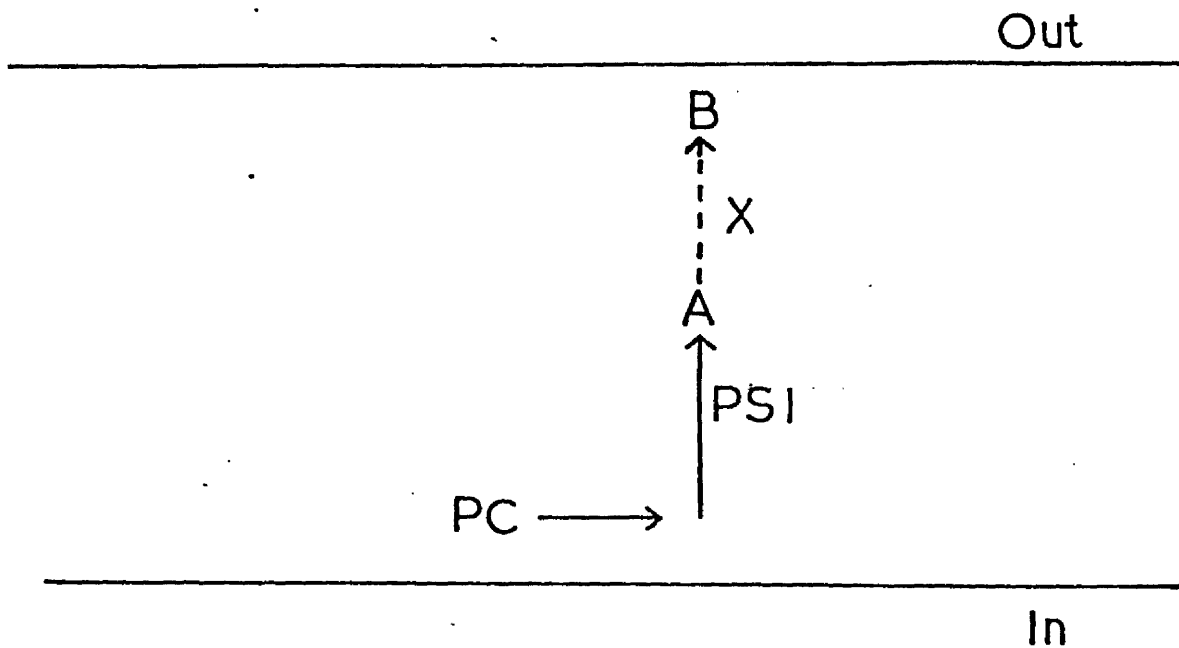
Thus the slow phase would result from the further separation of charge by secondary electron movement from the primary acceptor of a reaction centre inside the membrane to a secondary acceptor situated on or near the outer surface. Such a mechanism accounts for the second phase of the carotenoid band shift in photosynthetic bacteria (Jackson and Dutton, 1973) (see Figure 10a).

The second hypothesis may be that a separate charge separation across the membrane, through a different channel to that of the photochemical complex, may occur in response to the primary photochemical charge separation. This second movement of charge across the membrane might take the form of electrons moving from the inside of the membrane to the outside or protons moving from the outside inwards or a combination of the two (Joliot and Delosme, 1974) (Figure 10b).

The third type of mechanism recently postulated by Olsen and Barber (1981) suggests that the slow phase results not from further charge separation but merely from the conversion of the localised transmembrane electrical field, due to the fixed charges created by the initial separation, into a field due to diffusible charges. They suggest that, if the pigments responsible for the electrochromic absorption change are situated some distance from the reaction centre complex, the pigments will be exposed to a more intense electric field as it becomes delocalised into a uniform field over the whole membrane rather than when it first appears at the localised reaction centre.

Much of the experimental data acquired recently has been interpreted as evidence for the second of these hypotheses; that a second charge separation through a different channel is set off by the initial primary photochemical charge separation (see Velthuys, 1980a; Bourges-Bocquet, 1981; Crowther and Hind, 1980).

A



B

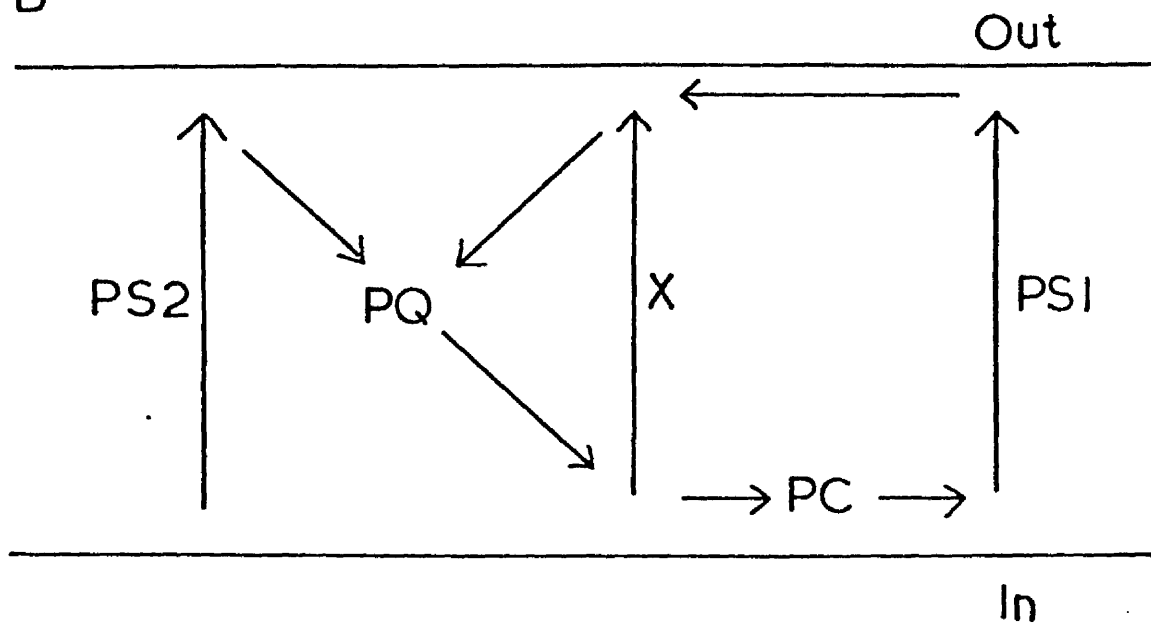


Figure 10. Schematic representation of the possible routes of electron movement (X) in the thylakoid membrane which result in a slow, small increase in charge separation observed as the slow phase of absorption increase at 515 nm (see text for explanation).

The site of this secondary charge separation is now the subject of much interest due to its possible connection with the site of electron driven proton translocation between the two photosystems. Hind's group (see Slovacek et al. 1979; Crowther and Hind, 1980, etc) has correlated the maximal extent of the slow phase with a number of the conditions favouring cyclic electron flow in chloroplasts such as its sensitivity to DBMIB, and antimycin A and poisoning by low concentrations of DCMU and dithionite (Crowther and Hind, 1980). The kinetics of cyclic electron flow through cytochrome b₅₆₃ and cytochrome f and the rise time and extent of the slow phase were also found to correspond (Slovacek et al. 1979). Others however have observed the slow phase during linear electron flow. Bourges-Bocquet (1977b) found that the slow phase was correlated with the reduction of cytochrome: f and plastocyanin in Chlorella. This reduction only took place following the first few exciting flashes and was inhibited by the quinone antagonist DBMIB. Furthermore, she showed that oxidants inhibited the slow phase but reductants, e.g. dithionite, induced a maximal extent. Thus the reduction of some component was indicated for the slow phase to be present. Velthuys (1978) observed that in chloroplasts the slow phase was associated with the oxidation of PQ which had been pre-reduced with light and thus the reductant necessary for the slow phase was in fact PQ. Bourges-Bocquet (1981) found however that in Chlorella the slow phase was present when PQ was only slightly (circa 12%) reduced and that another component, possibly another quinone, was the necessary component.

Velthuys (1978 and 1980a) also showed that the slow phase was observed under conditions where an extra proton was taken up during non-cyclic electron flow between the two photosystems. This view was supported by Farineau et al. (1980) and agreed with the conclusions of Fowler and Kok (1976) that more than one proton was taken up per electron transported from PSII to PSI.

Cytochrome b₅₆₃ has been implicated in the reaction leading to the slow phase. As described in section 1.3.3.5 the exact function and location of this component have been unclear but it had been thought to function in the cyclic pathway between ferredoxin and the intersystem carriers. Velthuys (1979) however showed that such a simple scheme was unlikely; both its reduction and oxidation rates were accelerated when the PQ pool was reduced, i.e. under similar conditions to those which induced the slow phase and led to the reduction of cytochrome f. This suggested that electron flow to the secondary donors of PSI alternately causes the reduction and oxidation of cytochrome b₅₆₃ whilst providing the conditions for the slow phase.

A number of schemes have been proposed to account for these various observations of cyclic and non-cyclic electron flow, of extra proton pumping, and of the generation of the extra electrogenic step (see Bourges-Bocquet, 1977b; Velthuys, 1979; Crowther and Hind, 1980). The most recent ones are based around the proton-motive "Q-cycle" of electron transport originally proposed by Mitchell (1975).

The Q-cycle is based on the idea that semiquinones are unstable to dismutation, which is the reduction of a molecule of semiquinone to the quinol by another molecule of semiquinone which is itself oxidised to the quinone. In Mitchell's scheme such a dismutation occurs not as a direct reaction but by the intermediate of a transmembrane chain of electron carriers (possibly cytochrome b₅₆₃). Thus PQ can take up a proton from the outer phase with an electron from either photosystem to form a semiquinone. A second proton may then be taken up with an electron via vectorial dismutation forming a quinol. The quinol is able to diffuse across the membrane and reduce cytochrome f (or the Rieske iron-sulphur centre) and release one proton to the inner phase forming the unstable semiquinone.

The semiquinone dismutates to the quinone releasing a second proton to the inner space and an electron which is transferred back across the membrane reducing the outer semiquinone to the quinol and the process then repeats itself. Thus two protons may be transported for each electron passing through PQ (see Figure 11). This scheme allows for an extra electrogenic step by way of the vectorial transport of an electron, possibly via cytochrome b₅₆₃, during the dismutation reaction. Although the Q cycle does not account for all of the observed phenomena (see Crowther and Hind, 1980; Malkin, 1982; Cox and Olsen, 1982) a modified Q cycle might. Crowther and Hind (1980) suggest an additional component V, with a redox potential of -55 mV is implicated in such a modified scheme, and may be the reductant proposed by Velthuys (1978) and Bourges-Bocquet (1977b) necessary for the slow phase. Bourges-Bocquet (1980) proposes that this component may be an additional iron-sulphur centre but such a component is at present only hypothetical.

The first part of this thesis is an investigation into the effects of a number of chemical and physical treatments on the slow phase of the absorption increase at 515nm in Chlorella. Observations were made on the flash-induced absorption changes at 515nm, oxygen evolution, prompt fluorescence inductions and the flash-induced absorption changes indicative of the redox reactions of cytochrome f and b₅₆₃.

It was hoped that these studies would lead to further information in elucidating the electrogenic mechanism of the slow phase and therefore on the secondary steps of electron transport and the resulting coupled proton pumping.

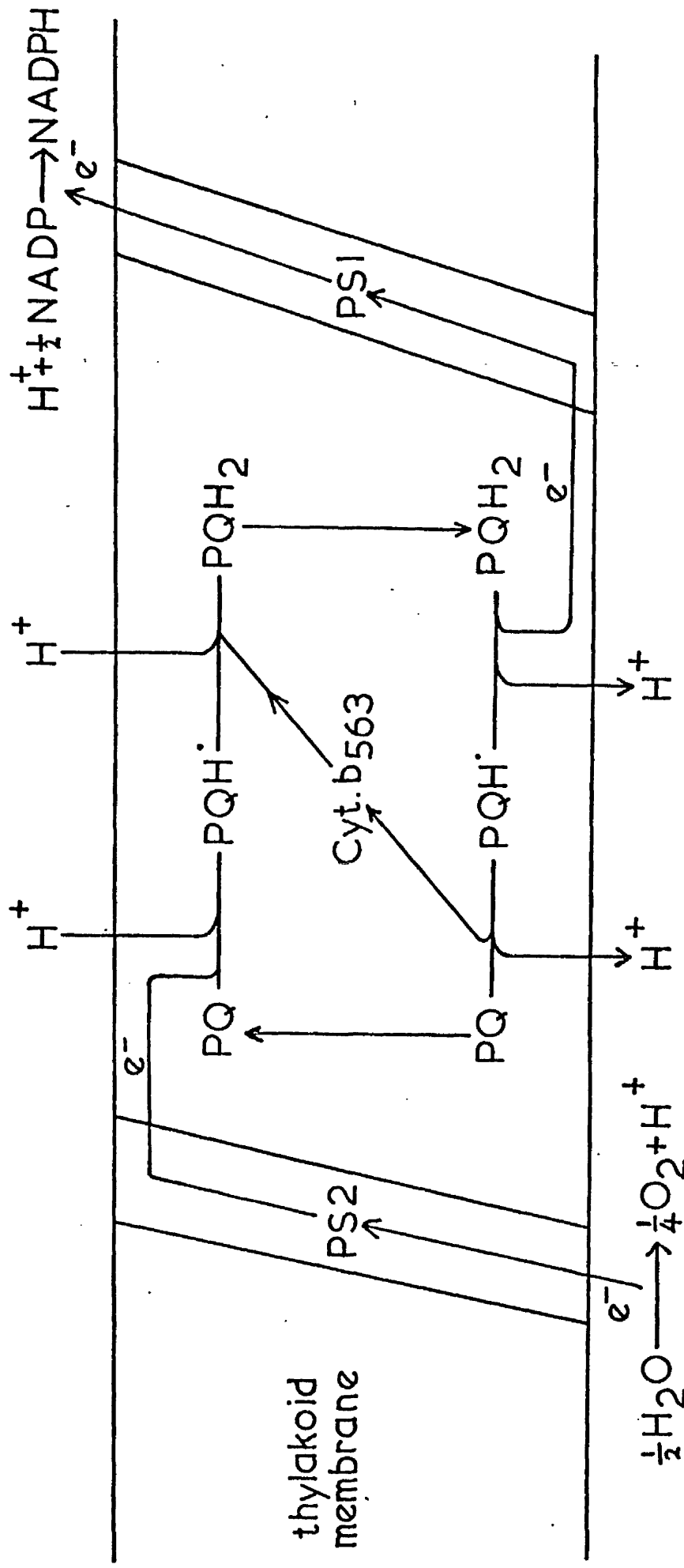


Figure 11. Schematic representation of vectorial electron transport in the thylakoid membrane incorporating a proton motive Q cycle allowing the vectorial dismutation of plastoquinone via cytochrome b_{563} . This results in the transfer of an extra proton per electron across the membrane and the possible extra charge separation via cytochrome b_{563} observed as the slow phase of absorption increase at 515 nm (adapted from Cox and Olsen, 1982).

1.6 The Effects of Heavy Metals on Photosynthesis

A number of the heavy metals are known to be necessary nutrients for the successful growth and development of plants and algae (see Walker, 1953), however, the levels of requirement are so small that they are called micronutrients. These heavy metal ions are necessary for the activity of metallo-enzymes (see Hewitt, 1958; Lehninger, 1975) or in specialised proteins such as superoxide dismutase, cytochrome oxidase and plastocyanin (see section 1.3). But heavy metals become increasingly toxic to plants and algae at higher concentrations. That heavy metals are poisonous towards microscopic marine life has been recognised for most of this century. Paints containing the oxides of heavy metals were used to prevent the fouling of surfaces immersed in water, by deterring the settlement of algal spores and plankton. Attempts were also made to try to control the "red tides" of dinoflagellates by adding heavy metal ores to the affected areas (Davies, 1978).

Thus until recently interest in the toxicity of the heavy metals towards plants had been more directed toward their use in prevention of unwanted growth. However, over the past twenty years there has been an increasing awareness that the build up of these elements in our environment from various sources such as industrial effluents, sewage, mine-run off, smelters, agriculture and waste disposal could be having a serious deleterious effect upon the growth and development of the organisms, particularly the primary producers, in these areas.

The heavy metal build up leads firstly to a decrease in productivity of the primary producers and secondly provides an entry into the food chains of the ecosystems, of which the higher trophic levels are often used for human consumption.

A great deal of concern is being shown towards the increasing levels of heavy metals in both our terrestrial and aquatic environments and their effects upon the primary producers in those environments. Direct chemical analysis of water samples has provided one method for monitoring heavy metal levels in aquatic environments but it is not economical and the results do not necessarily indicate their effects on living organisms. Thus, biological indicators such as teleosts and macroalgae have been used as bioassays (Wong, 1980). However, these multicellular aquatic organisms are not the most satisfactory biological indicators as they possess complex homeostatic mechanisms for dealing with environmental changes and thus may survive under conditions lethal to other organisms (Hynes, 1960). Unicellular organisms are therefore preferred for their simpler, more easily observed reactions and life cycles and their more direct response to suboptimal living conditions. Thus, microalgae have been used for detecting and assaying the levels of heavy metals in a number of aquatic environments (see Wong, 1980; Davies, 1978; Whitton, 1979).

It can, therefore, be seen that an investigation into effects of heavy metals on microalgae is an important topic both because of the decrease in productivity and also as they provide a method of monitoring environmental levels of the heavy metals. Until recently studies on the effects of heavy metals had been limited to the effects on overall growth of phytoplankton populations but recently it has become obvious that photosynthetic carbon fixation is one of the processes most sensitive to heavy metals and therefore a detailed investigation of the effects of heavy metals on photosynthesis is warranted.

The second part of this thesis is an investigation of the short-term incubation effects of four heavy metals on the light reactions of photosynthesis. Two of these metals; copper and zinc, are essential micronutrients and the others; lead and mercury are not. A number of recent studies on the effects of heavy metals have been conducted on chloroplasts isolated from higher plant tissues, particularly spinach and these studies yielded a number of possible sites of actions of the heavy metals. However, these in vitro studies may not be completely relevant physiologically as they do not take into account a number of factors present in whole cells and tissues. For instance, not only are concentrations of soluble metals in cell cytoplasm unknown but the activity of a given metal in the cytoplasm is affected by chelating carboxylic and amino acids, by cations other than the metal in question, and by pH (Foy et al. 1978).

In order to bridge the gap between the in vitro studies on chloroplasts and the ecological studies on phytoplankton, the fresh water alga Chlorella was used as a suitable intermediate in the studies on heavy metal inhibition of photosynthesis. It was hoped that in vivo studies with Chlorella would help to characterise the physiological mode of inhibition of photosynthesis by the four heavy metals studied.

The following is a brief summary of the relevant previous investigations on the influence of the four heavy metals copper, zinc, mercury and lead on photosynthesis.

1.6.1 Copper

Records of the use of CuSO_4 as an algicide date back to the beginning of the century when it was used to control Spirogyra (Moore and Kellerman, 1904).

The first major investigations describing the influence of copper on the rate of photosynthesis were conducted by Greenfield (1942) in Chlorella vulgaris and by MacDowall (1949) in isolated chloroplasts. They concluded that both the light and dark reactions of photosynthesis were inhibited by $0.1 \mu\text{M}$ Cu^{2+} in Chlorella vulgaris and $100 \mu\text{M}$ in chloroplasts. Steeman-Nielsen and Wium-Anderson (1971) reported that the unicellular diatom Nitzschia palea was even more strongly effected than Chlorella vulgaris. Steeman-Nielsen et al. (1969) repeated most of these early experiments in the first thorough investigation of copper on photosynthesis in Chlorella pyrenoidosa. They concluded that a large number of factors influenced the amount of inhibition; the duration of exposure, the light intensity during the exposure and under which the algae had been grown, the cell concentration (Cu^{2+} to chlorophyll ratio) and the composition of the experimental medium. To a lesser extent the temperature and developmental state of the exposed culture also affected the Cu^{2+} inhibition as did the pH and anaerobic condition of the culture. Furthermore, two different types of Cu^{2+} effect occurred; in a solution of CuSO_4 only, a fast inhibition occurred (minutes) whereas in a balanced nutrient solution no inhibition occurred until after 4-6 hours and the algae had to be illuminated for an effect to occur, suggesting the second case of inhibition of photosynthesis was due to an indirect effect of copper. This might be explained by the observation that copper inhibits the liberation of autospores which would lead to the accumulation of excess photosynthetic products by the cells and thus decrease the rate of photosynthesis (Steeman-Nielsen et al. 1969). McBrien and Hassell (1965) were the first of a number of observers to report that Cu^{2+} uptake was associated with the release of cell K^+ in Chlorella (see also Steeman-Nielsen and Wium-Anderson 1971; Overnell, 1975b).

This suggested that the primary effect of Cu^{2+} toxicity was damage to the cell membrane resulting in loss of cell contents, but this is now questionable because cells which have lost a considerable amount of potassium are still capable of subsequent growth (Shioi et al. 1978b). But a great deal of evidence does suggest that Cu^{2+} causes the destruction of membranes and their constituents. Lipid destruction of the cell membrane by Cu^{2+} was first suggested by Gross et al. (1970) who found that copper ions caused a decrease in the packed cell volume and pigment destruction in Chlorella. Samuelsson and Oquist (1980) showed a rapid destruction of chlorophyll-protein complexes in the light by Cu^{2+} . The P700 chlorophyll-protein complex of PSI was more sensitive to Cu^{2+} than the light-harvesting complex suggesting a preferential destruction of chlorophyll a. They suggested that the underlying mechanism for this lipid destruction could be the formation of H_2O_2 in a Cu^{2+} catalyzed peroxidation. Sandmann and Böger (1980 a and b) have correlated a Cu^{2+} -induced decrease in photosynthesis and growth with light-induced lipid peroxidation in the photosynthetic membrane both in the unicellular green alga Scenedesmus acutus and spinach chloroplasts. The peroxidation is itself a result of the inhibition of electron transport of both photosystems by Cu^{2+} resulting in the production of the highly destructive hydroxyl radical, OH^\bullet (Sandmann and Böger, 1980a).

Investigations of copper effects on electron transport reactions have been attempted in whole cells of Chlorella (Cedeno-Maldonado and Swader, 1974) and Scenedesmus acutus (Bohner et al. 1980), in broken cells of the unicellular green algae Ankistrodesmus falcatus (Shioi et al. 1978b) and in isolated chloroplasts (Cedeno-Maldonado et al. 1972; Shioi et al. 1978a; Vierke and Struckmeier, 1977; Samuelson and Oquist, 1980). Electron transport inhibition has been reported for both photosystems although it is generally assumed that the most sensitive site is on the oxidising side of PSII.

There is disagreement as to the actual site of inhibition; Shioi et al. (1978a) and Cedeno-Maldonado et al. (1972) suggest Cu^{2+} inhibits near to the PSII reaction centre but Vierke and Struckmeier (1977) propose a site much nearer the water splitting complex.

The second site of Cu^{2+} inhibition is thought to be on the reducing side of PSI. Shioi et al. (1978a) reported that Cu^{2+} inhibits the reduction of NADP and cytochrome c by directly inactivating ferredoxin, possibly by the oxidation of the sulphhydryl groups. The actual mechanism of inactivation at these two sites is unknown at present. Vierke and Struckmeier (1977) suggest that Cu^{2+} binds to a membrane protein which is not involved in electron transport itself, but which causes structural changes inside the membrane upon Cu^{2+} binding with the subsequent inhibition of electron transport.

Recently a third site of inhibition has been suggested by Uribe and Stark (1982). They have reported that both cyclic and non-cyclic phosphorylation in spinach chloroplasts are strongly inhibited by Cu^{2+} of micromolar concentration. The proposed site of inhibition is the CF_1 coupling factor and it is thought that Cu^{2+} causes an oxygen independent oxidation of sulphhydryl groups on the coupling factor. In addition, they observed that at higher concentrations the inward pumping of protons was inhibited but these concentrations were far higher than those which were necessary to inhibit the coupling factor.

1.6.2 Mercury

Like copper the toxic effects of mercury compounds have been known for some time and the susceptibility of primary producers to these extremely toxic metals is now of great concern. Concentrations as low as $2.5 \times 10^{-9}\text{M}$ reduce the growth of green algae and inhibit cell division. Higher concentrations ($2.5 \times 10^{-8}\text{M}$) can inhibit photosynthesis of phytoplankton populations by at least 70% (see Stratton et al. 1980).

Two of the first reports of the toxic effect of mercury on photosynthesis were by Greenfield (1942) and MacDowall (1949). The former found that $2.5 \times 10^{-5} \text{M}$ Hg^{2+} completely inhibited photosynthesis in Chlorella vulgaris possibly by destroying the light harvesting pigments. MacDowall (1949) on the other hand showed a highly specific inhibition of photosynthesis in the dark in isolated chloroplasts. A more recent study by Kamp-Nielsen (1971) on Chlorella pyrenoidosa showed that the effects of Hg^{2+} were similar to those caused by Cu^{2+} but with a number of differences; Hg^{2+} generally acted at lower concentrations and its inhibition was not counteracted by other ions, it prevented cell division but without the concomitant accumulation of assimilation products seen with Cu^{2+} and, in agreement with Greenfield's results (1942) it disrupted the photosynthetic pigments.

Like Cu^{2+} , Hg^{2+} has been shown to induce a light-dependent leakage of K^{+} ions through the cytoplasmic membrane (Kamp-Nielsen, 1971; Shieh and Barber, 1973; Overnell, 1975b). However, the concentrations which inhibited photosynthesis were an order of magnitude less than that which induced K^{+} leakage (Overnell, 1975b) and therefore a direct relationship between the two phenomena is questionable. Shieh and Barber (1973) further observed that low concentrations of Hg^{2+} actually stimulated K^{+} uptake in an energy dependent reaction suggesting the Hg^{2+} interacts directly with the K^{+} transporting system as well as causing a breakdown in the membrane structure. Stratton et al. (1980) noted a more devastating effect of Hg^{2+} on the membranes of the cyanobacterium Anabaena inaequalis. They found $5 \times 10^{-7} \text{M}$ Hg^{2+} caused total lysis of all the cell membranes within one hours incubation.

A number of specific effects, of Hg^{2+} and organic mercurials, on photosynthetic electron transport and phosphorylation has been reported in recent years. Kimimura and Katoh (1972) showed that Hg^{2+} at a molar ratio of Hg^{2+} to chlorophyll of one inhibited electron transfer from cytochrome f to the reaction centre of PSI, P700, indicating a block at the plastocyanin site. Specifically they proposed that Hg^{2+} attacked plastocyanin at the cupro-sulphydryl linkage and replaced Cu^{2+} with Hg^{2+} .

These conclusions would seem to be supported by Radmer and Kok (1974) who showed that Hg^{2+} induced a damping of the flash oxygen yields and that ferricyanide restored the flash yield pattern to normal. They proposed that Hg^{2+} specifically blocked the re-oxidation of the PSII electron acceptor via the intersystem electron transport chain. Miles et al. (1973) on the other hand proposed that HgCl_2 (100 μM) inhibited electron transport, in spinach chloroplasts, by acting as a preferred electron acceptor at the site of the quencher (Q) of chlorophyll fluorescence in PSII.

Higher concentrations of Hg^{2+} (Hg^{2+} : chlorophyll = 1.4) and organic mercurials (200 μM) have been shown to inhibit PSII. Kimimura and Katoh (1972) proposed that Hg^{2+} inhibited PSII by acting on the pigment systems or reaction centres of the two photosystems as revealed by a suppression of the photooxidation by P700 and decreased fluorescence yield in the presence of DCMU or dithionite (see section 1.5.2). Honeycutt and Krogmann (1972) suggested that mercury acted, at a site similar to copper, between the H_2O splitting complex and the reaction centre of PSII.

Components on the reducing side of PSI such as ferredoxin and ferredoxin-NADP-reductase have also been shown to react with mercurials (Tagawa and Arnon, 1965).

Honeycutt and Krogmann (1972) showed that phenylmercuric acetate inhibits ferredoxin specifically and thus NADP reduction in spinach chloroplasts, whereas Zanetti and Forti (1969) showed that mercurials such as p-hydroxymercuribenzoate and p-chloromercuriphenylsulphonate inactivate the enzyme ferredoxin-NADP-reductase, particularly in the presence of NADP, by reacting with the sulfhydryl groups. Whether inorganic Hg^{+2} acts at this site is unclear. Low concentrations of mercuric acetate and chloride (less than 1 μ mole Hg^{2+} ; mg chlorophyll) have been shown to act as an energy transfer inhibitor (Izawa and Good, 1969; Bradeen et al. 1973). That is to say that it blocks phosphorylation by interfering with the terminal enzymatic steps of ATP synthesis in this case probably by binding to the coupling factor CF_1 via sulphhydryl residues on the γ subunit (McCarty and Fagen, 1973).

1.6.3 Zinc

In comparison to copper, mercury and lead very little work has been carried out on the toxic effect of zinc on photosynthesis. Although Zn^{2+} is essential in trace quantities as a component of many enzyme systems (Bowen, 1966) at higher levels it becomes increasingly toxic. Greenfield (1942) was again responsible for first showing that Zn^{2+} inhibited photosynthesis. He observed that far higher concentrations (circa $10^{-1}M$) were necessary though, than for Cu^{2+} , Co^{2+} or Hg^{2+} to inhibit photosynthetic oxygen evolution in Chlorella vulgaris. These levels might be considered to be too high to warrant environmental concern, however Davies and Sleep (1979) have reported that river drainage from mineral bearing, and industrialised, regions has produced elevated concentrations of heavy metals in several parts of the coastal waters around the UK and other European countries. They report that these levels are high enough to cause reduction of primary production in such areas. They showed that increasing levels of Zn^{2+} up to 1.2 μ M inhibited the fixation of C^{14} by up to 25% in phytoplankton populations.

Overnell (1975b and 1976) reported that concentrations higher than 1 mM Zn^{2+} were necessary before photosynthesis in the marine, unicellular green algae Dunaliella tertiolecta was inhibited even slightly but other species were more sensitive, i.e. the unicellular marine algae Monochrysis lutheri. Furthermore Zn^{2+} toxicity differed from Cu^{2+} and Hg^{2+} toxicity in that no K^{+} leakage through the cell membrane was observed.

Wong (1980) investigated the effect of Zn^{2+} on the growth of Chlorella pyrenoidosa in hard and soft water and concluded that levels of greater than $5 \times 10^{-5}\text{M}$ Zn^{2+} were necessary to inhibit the growth rate but the lethal effect was decreased in hard water containing 4 mM CaCO_3 .

The only major report aiming to characterise the mode of inhibition of photosynthesis by Zn^{2+} salts was recently reported by Tripathy and Mohanty, (1980). They studied the effects of ZnSO_4 on electron transport and chlorophyll fluorescence in chloroplasts isolated from barley. Zn^{2+} concentrations from 0.5 mM upwards inhibited overall electron transport and PSII-supported electron transport but concentrations above 5 mM were necessary to prevent electron flow through PSI. They suggested that Zn^{2+} inhibits photosynthesis between the water splitting complex and the site of electron donation by NH_2OH before the PSII reaction centre (see section 1.3). In contrast to the washing of Hg^{2+} treated chloroplasts (Kimimura and Kato, 1972) washing of Zn^{2+} treated chloroplasts restored the oxygen evolving capacity. The inhibition of PSI at high concentrations was not further investigated.

1.6.4 Lead

The concentration of lead in our environment has been increasing dramatically over the past century mainly due to its liberation from exhausts of motor vehicles and its widespread industrial use (Chow and Earl, 1970) but also from run off from lead mines in certain areas (Welsh and Denny, 1976). Although the effects of lead have been studied on various animal tissues and enzymes, and also on plant transport systems, there has been little detailed work reported on the effect of this metal on the physiology of plants (Bazzaz and Govindjee, 1974).

Lead has been shown to inhibit the growth of various marine algae (Hessler, 1974) and freshwater green algae (Monahan, 1976; Whitton, 1970) at concentrations as low as $1.9 \times 10^{-5}M$ although the inhibition was sensitive to phosphate availability of the medium as well as the pH (Monahan, 1976). Overnell (1975 a and b) found the Pb^{2+} inhibition of photosynthesis as measured by oxygen evolution was species dependent; the freshwater green alga, Chlamydomonas reinhardtii was 50% inhibited by $1.5 \times 10^{-6}M$ Pb^{2+} whereas the marine alga, Dunaliella tertiolecta needed $10^{-4}M$ Pb^{2+} for 50% inhibition. He further showed that, unlike Cu^{2+} and Hg^{2+} , Pb^{2+} caused no K^{+} leakage across the cell membranes of these algae (Overnell, 1975b).

Miles et al. (1972) were the first to show a Pb^{2+} -induced inhibition of electron transport in isolated chloroplasts. They deduced that only PSII was inhibited at millimolar concentrations and PSI was not inhibited by Pb^{2+} concentrations as high as 2.4 mM. Their studies on fluorescence indicated the specific site of inhibition to be between the water splitting complex and the primary donor of PSII. Bazzaz and Govindjee (1974) confirmed this result and suggested that Pb^{2+} inhibition occurred at the same site as Tris washing (see section 1.3.1).

They also suggested, from fluorescence studies, that Pb^{2+} induces structural changes in the thylakoid membrane leading to changes in the energy distribution between PSII and PSI (see section 1.5.2) in a fashion opposite to low concentrations of divalent cations (Murata, 1969) but similar to low concentrations of monovalent cations (Gross and Hess, 1973). Wong and Govindjee (1976) studied the primary reactions of PSI and found that high concentrations of Pb^{2+} (7.2 mM) partially inhibited the photo-oxidation of P_{700} and also altered the kinetics of the rate of electron transport to P_{700} or its primary donor. Homer et al. (1980) showed a different fluorescence effect; they correlated Pb^{2+} inhibition (circa 2 mM) as measured by oxygen evolution with an increase in fluorescence emission attributed to inhibition of PSII. Further metal addition (above 2.5 mM) caused a decrease in fluorescence, which they explained as heavy metal quenching of chlorophyll fluorescence within the membrane (Homer et al. 1980).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cultures and Harvest of Chlorella

The unicellular green alga Chlorella emersonii (Cambridge Centre of Algae and Protozoa List No. 211/8b) was used as the major experimental material for this study. The cells were cultured in 300ml of growth medium (see appendix for all culture and isolation media) in 500ml Dreschel bottles at room temperature. The cultures were bubbled with air and agitated by means of a magnetic stirrer to prevent sedimentation and aggregation of the non-motile cells. Continuous illumination was provided by a Crystelco 40W "Warm White" fluorescent tube giving a light intensity of approximately 7Wm^{-2} at the bottle sides. The algal cells were ready to be harvested after 3-5 days at which point they were fairly concentrated whilst within the log phase of growth (see Figure 12 for growth curve).

The chlorophyll concentration of the culture was determined spectrophotometrically from a methanol extract of the cells (Holden, 1965). 5mls of the cell suspension was centrifuged for 10 minutes at $4000 \times g$ in an MSE Super Minor centrifuge to harvest the algal cells.

The pellet was then homogenised in 10mls of methanol and incubated for 10 minutes at 50°C . The suspension was shaken frequently until all the pigment was extracted and then filtered. The absorbance of the filtrate was measured at 650nm and 665nm, using methanol as a blank, in an MSE Spectroplus and the concentration calculated, after correction for the dilution factor, as follows:

$$\text{Total Chlorophyll concentration} = (25.5 \times \text{OD}_{650}) + (4.0 \times \text{OD}_{665}) \text{ (mg ml}^{-1}\text{)}$$

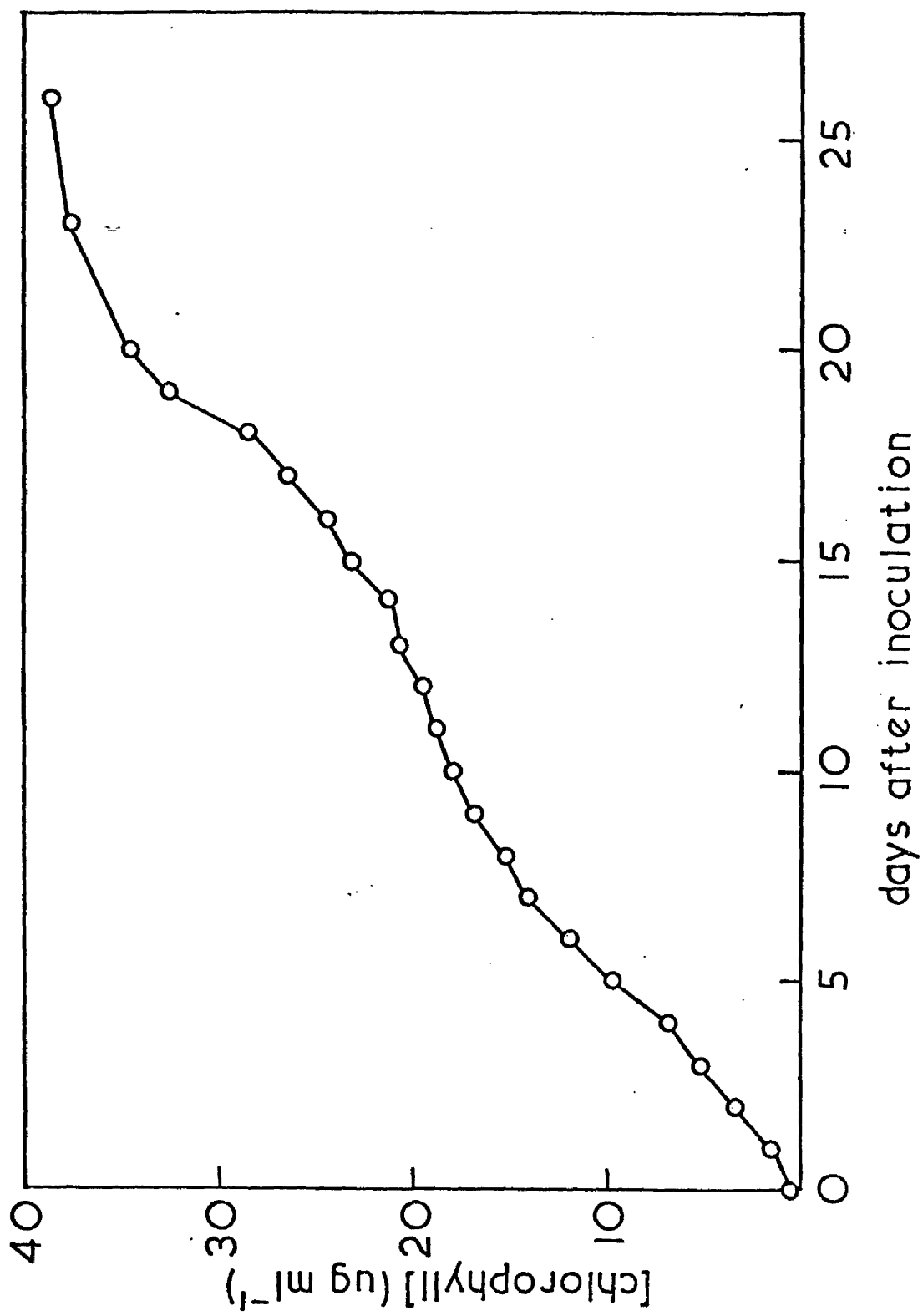


Figure 12. The growth curve of *Chlorella emersonii* after an initial inoculation of 15 ml of 3-5 day old cells.

A standard curve (Figure 13) was constructed of total chlorophyll concentration, determined as above, against the apparent absorption (which includes light scattering) at 682nm. This was determined in a Pye Unicam SP8000 scanning Spectrophotometer using a blank of distilled water and setting the trace to zero at 850nm. Thus the chlorophyll concentration of an algal suspension could be determined rapidly by just measuring its apparent absorption at 682nm and referring to the standard curve.

The algal cells were harvested by centrifugation at 4000 x g for 5 minutes in an MSE Super Minor bench centrifuge. The algal pellet was either resuspended in growth medium to give a final chlorophyll concentration of 30µg ml⁻¹ for the studies of the 515nm change or washed and resuspended in 0.01M MOPS (morpholinopropane-sulphonic acid), pH 6.8, at a chlorophyll concentration of 5µg ml⁻¹ for the studies on heavy metal toxicity.

To prevent rapid sedimentation of the cells in the cuvette during absorption and fluorescence measurements, the viscosity of the resuspension medium was increased by adding 7% Ficoll. Ficoll is a high molecular weight (circa 400 000) polymer of sucrose and thus a 7% solution will not appreciably lower the water potential of the medium. In addition, it has been reported to not interfere with the physiological properties of the algae (Joliot and Delosme, 1974).

2.2 Isolation of Chloroplasts

2.2.1 Intact Chloroplasts

Spinach (Spinacia oleracea, var. Dominant) (supplied by Nutting and Thoday, Cambridge) was germinated in vermiculite in a plant growth chamber at 25°C with a 12 hour photoperiod.

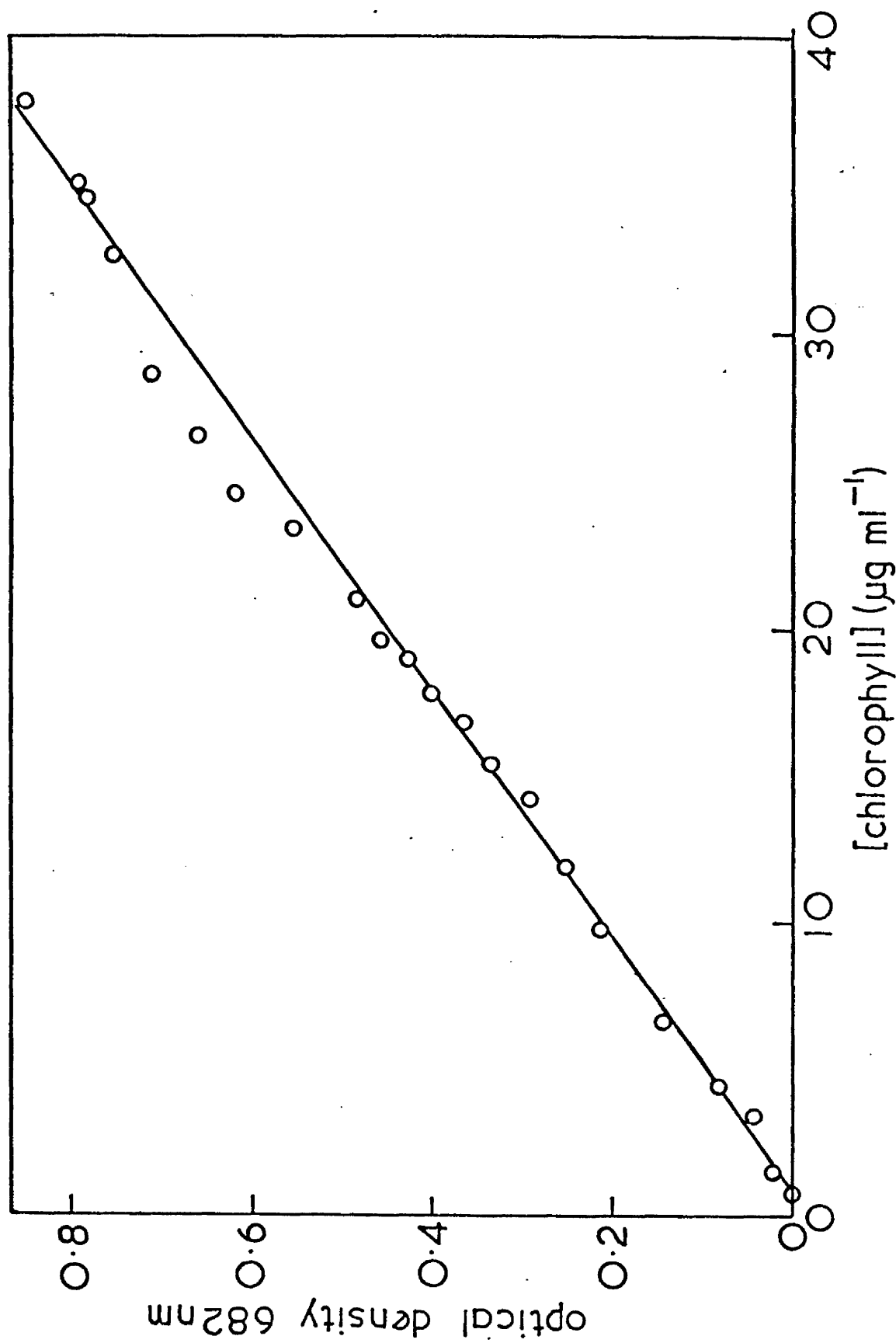


Figure 13. A standard curve of total chlorophyll concentration versus the apparent absorption at 682 nm for Chlorella.

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Illumination was provided by a bank of white fluorescent tubes (Thorn 20W 3500) giving a light intensity of 14 Wm^{-2} at the top of the plants. The seedlings were watered every day with the nutrient solution described in appendix 1 (Baker et al. 1978). When they were approximately 2 inches high the seedlings had thin strips of polystyrene wrapped round their stems allowing them to float on an aerated liquid culture of nutrient medium where growth was continued under similar conditions of light and temperature. The leaves were harvested after approximately 6 weeks (Baker et al. 1978).

Peas (Pisum sativum, var. Feltham First) (supplied by Charles Sharp & Co. Ltd.) were grown in moist vermiculite under the same environmental conditions as the spinach. The plants were harvested after approximately 14 days.

Intact and osmotically shocked chloroplasts from pea and spinach were isolated essentially by the method of Stokes and Walker (1971). Approximately 60g of deribbed spinach leaves or destemmed pea leaves were placed in a pre-cooled perspex grinding vessel containing 200ml of ice-cold grinding medium (see appendix 1). The mixture was homogenised for two 5 second bursts at speed setting 6, using a Polytron PVC-2 homogeniser. The resulting homogenate was squeezed through two layers of muslin and then filtered through a further eight layers containing a thin layer of cotton wool. The filtrate was centrifuged immediately in pre-cooled tubes using an MSE Super Minor centrifuge at $4000 \times g$ for 30 seconds and then braked as quickly as possible to minimise contamination by broken chloroplasts. The supernatant was discarded and the loose pellet top and sediment on the tube sides carefully washed and removed with a tissue to decrease contamination by broken chloroplasts. The pellet was then carefully resuspended with a paint brush in assay medium and stored on ice in the dark.

Intactness of the outer envelope of the chloroplasts was assayed using the method cited by Heber and Sautarius (1970). It is assumed that the chloroplast envelope is not permeable to potassium ferricyanide. Thus if the normal carbon fixation is inhibited by the addition of D-L-glyceraldehyde there will be no terminal electron acceptor, no electron transport, and consequently no oxygen evolution in intact chloroplasts. When the outer envelope is ruptured ferricyanide becomes available as an electron acceptor and oxygen is evolved. Therefore the ratio of oxygen evolution rates before and after osmotic shocking of the chloroplast suspension gives the proportion of broken chloroplasts in a sample. From this the proportion of intact chloroplasts in the sample can be calculated. The uncoupler NH_4Cl was added to maximise the rate of electron transport. This is necessary as shocked chloroplasts lose all the soluble unbound components of the Calvin cycle and coenzymes of phosphorylation which would lead to artificially low rates of electron transport.

2.2.3 Osmotically Shocked Chloroplasts

When necessary osmotically shocked chloroplasts were prepared by shaking intact chloroplasts in an aliquot of water (or 10mM MgCl_2 in 1mM TRIS, pH 8.2 in the experiments indicated) for approximately 20 seconds followed by an addition of an equal volume of double strength assay medium.

Chlorophyll concentration was determined by the method of Arnon (1949). This involved extracting the pigments by shaking 25 μl of the chloroplast suspension in 10ml of 80% Acetone. The resulting suspension was filtered and its absorption measured at 652nm. At this wavelength chlorophyll a and b make equal contributions to the absorption. After being corrected by a dilution factor the total chlorophyll concentration was determined by the equation.

$$\text{Total chlorophyll conc.} = \frac{OD_{652}}{34.5} \text{ (mg ml}^{-1}\text{)}$$

The chlorophyll concentration in all experiments using chloroplasts was approximately 35 $\mu\text{g ml}^{-1}$

2.3 Culture of Photosynthetic Bacteria and Preparation of Chromatophores

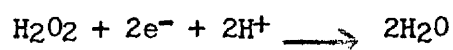
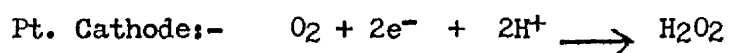
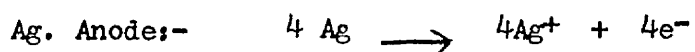
The photosynthetic bacteria Rhodospseudomonas capsulata strain Z1 and Rhodospseudomonas sphaeroides strain ATH2.4.1. were grown in a nutrient medium with succinate as the sole carbon source (see appendix 1). The cultures were grown from agar 'stabs' topped up with succinate medium and placed in the illuminated growth chamber at 27°C. When the cells had grown strongly into the medium they were transferred into 300ml of medium in flat sided bottles. During the transfer the bottle tops were flamed to prevent air-borne contamination. These cultures were then illuminated for 2-3 days by banks of 3 x 100 W incandescent bulbs approximately 40cm from the cultures giving an intensity of approximately 35Wm⁻².

The bacterial cells were harvested in an MSE 6L "Coolspin" centrifuge at 2400 x g for 1 hour and 40 minutes. They were then washed in 100mM KCl, 20mM MES.HCl, pH 6.5 and centrifuged at 17 000 x g for 15 minutes in an MSE 18 centrifuge.

To obtain the photosynthetic membranes the cells were first homogenised with small amounts of MgCl₂ and DNAase. The cells were then broken by passing them through a pre-cooled Aminco French Pressure Cell at approximately 10 ton p.s.i. This induces the invaginations of the intracytoplasmic membrane to "pinch off" forming sealed, round vesicles called chromatophores. The unbroken cells and large debris were removed by centrifugation at 17 000 x g for 15 minutes in an MSE 18 centrifuge. The remaining supernatant was spun at 100 000 x g for 1 hour 30 minutes in an MSE 50 centrifuge to obtain the chromatophores. The pellet was resuspended in a small volume of 40mM TRIS.HCl, pH 8.0.

2.4 Measurement of Steady-State Oxygen Evolution

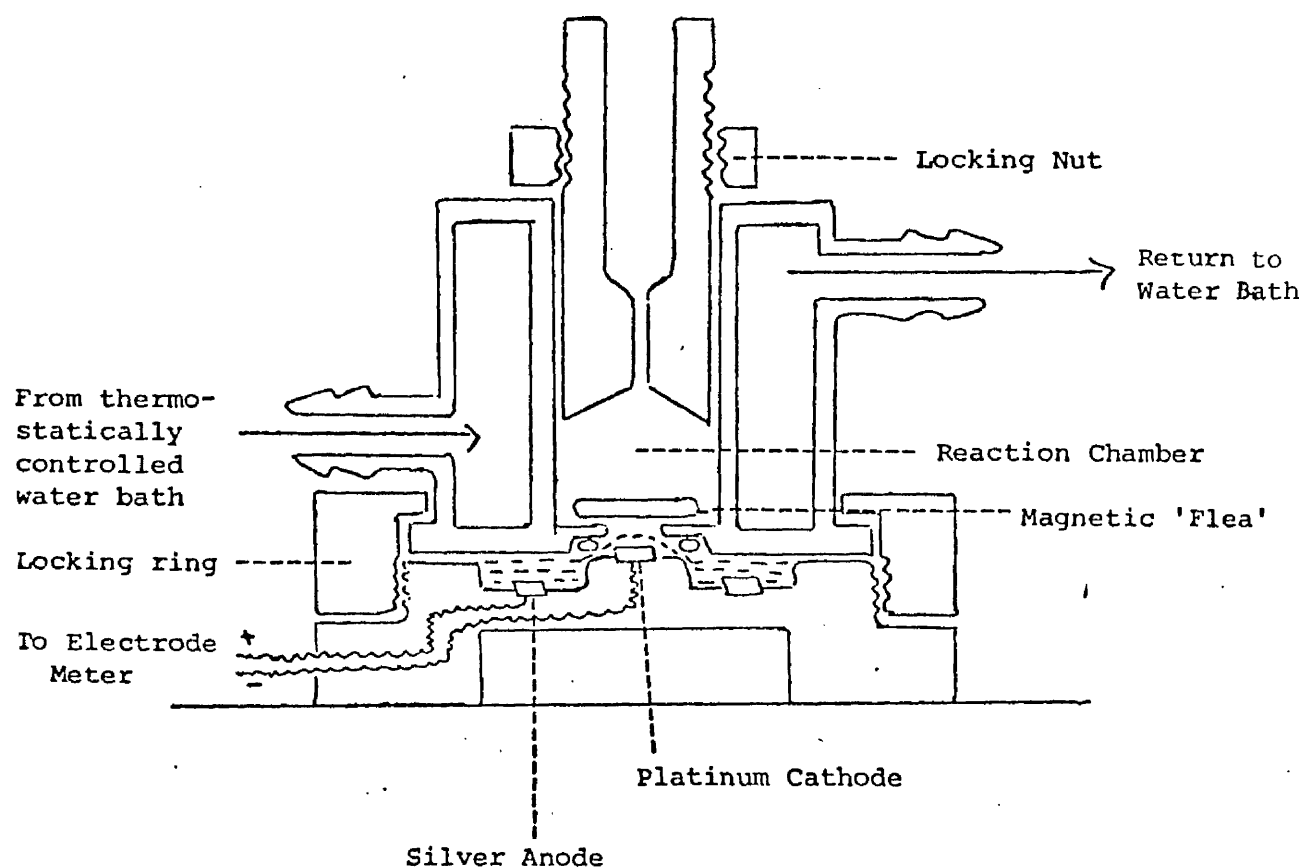
Light-dependent, steady-state oxygen evolution was determined polarographically in a modified Clark-type electrode (Figure 14) as adapted by Delieu and Walker (1972)(Rank Bros, Cambridge). It consists of an electrochemical cell separated by a thin oxygen permeable Teflon membrane from a reaction chamber containing the sample. The sample is continuously stirred by a miniature magnetic stirrer. The electrochemical cell contains a silver anode and a platinum cathode in a solution of saturated KCl. When a small electrical potential (0.65v) is applied across the electrodes the following electrochemical reaction occurs:



Therefore the current flowing through the cell is directly proportional to the oxygen concentration. Changes in the oxygen concentration of the reaction chamber brought about by photosynthesis or respiration are transmitted through the membrane into the cell and cause the current to alter accordingly (see Fork, 1972). This current was amplified and recorded on a Servoscribe chart recorder.

The whole unit was kept at approximately 23°C by a constant temperature water jacket and illumination was provided by a tungsten microscope bulb producing an irradiance of approximately 100 Wm⁻² at the cell surface. To prevent oxygen entering from the atmosphere the cell is sealed apart from a small hole through which small quantities of reagents can be added by microsyringe.

Figure 14. A diagram of the oxygen electrode used for steady-state oxygen evolution measurements.



2.5 Measurement of Flash-Induced Oxygen Evolution

Although the Clark type oxygen electrode is suitable for measuring the rate of steady state oxygen production by algae or chloroplasts its use is limited by the rate of diffusion of oxygen from the photosynthetic material to the platinum electrode. Thus for measuring oxygen evolved during fast, flash-induced, transitory phases of photosynthesis activity a modified electrode is used whereby the photosynthetic material is actually deposited on the platinum electrode. Any oxygen produced will be detected almost instantaneously by such a technique.

The electrode employed is similar to that described by Joliot and Joliot (1968) (see Figure 15). It consists of two compartments separated by a dialysis membrane. The bottom of the lower compartment is a bare platinum electrode upon which a drop of photosynthetic material (chlorophyll concentration circa $100 \mu\text{g ml}^{-1}$) is placed and is kept in place by being squeezed against the dialysis membrane. The compartment above the membrane contains the silver electrode as a ring lining its sides and is sealed above by a perspex window which acts as a light guide to the photosynthetic material.

The compartment is also provided with entrance and exit channels to allow a gravity fed flow of buffer containing 30mM KCl to pass slowly through the upper compartment and renew the solute content of the lower compartment through the dialysis membrane. When a polarising voltage of -0.65v is applied across the cell, the current generated is linearly proportional to the dissolved oxygen as in the Clark type electrode (see section 2.4). The signal was amplified, and when necessary, electronically differentiated for an increased signal to noise ratio. It was stored in a Datalab 901 transient recorder and read out onto a Servoscribe chart recorder. Actinic illumination was provided by a General Radio "Stroboslave" xenon flash lamp set on 'high', with an approximate duration of $5 \mu\text{sec}$ and a flash frequency of 2 or 4 Hz.

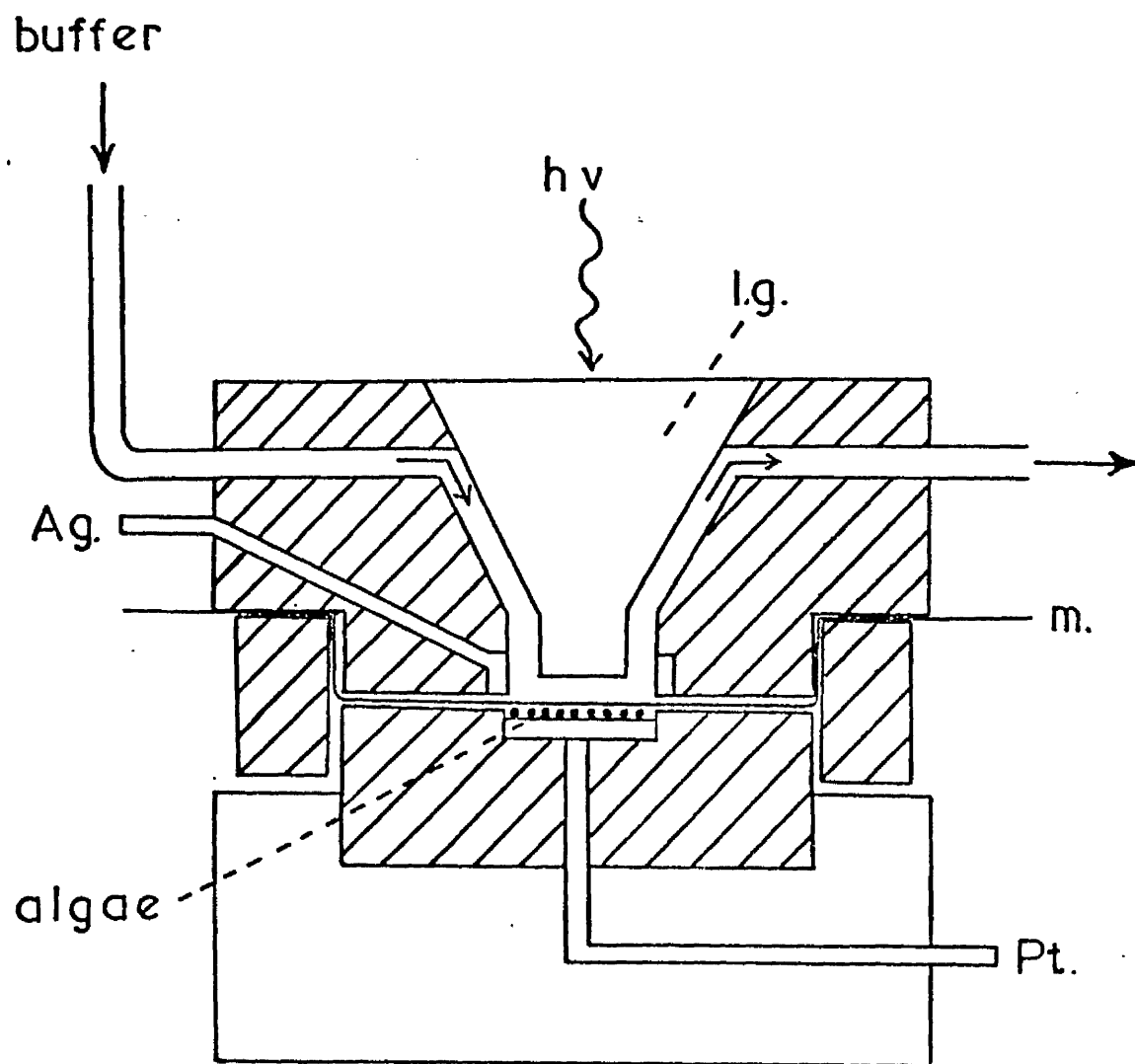


Figure 15. A diagram of the rapidly responding oxygen electrode used for measuring small amounts of oxygen evolved during a series of brief saturating flashes. Ag and Pt are the silver anode and platinum cathode; l.g. the perspex light guide; and m. the dialysis membrane.

2.6 Measurement of Flash-Induced Absorbance Changes

Flash-induced absorbance changes were detected in a rapidly responding single beam spectrometer (figure 16). A monochromatic weak monitoring beam was isolated from the output of a 130W tungsten-halogen projector bulb by an Applied Photophysics M380 monochromator. The entrance slit width was typically 2.5mm and the exit slit width set to give a half band width of 5nm. The intensity of the beam was kept to a minimum (approximately 0.02 Wm^{-2}) to prevent the monitoring beam acting in an actinic manner. The beam was passed through the sample contained in a 1cm pathlength quartz cuvette and detected by an EMI 9659 extended S20 photomultiplier tube powered by a Brandenburg 475R H.T. supply. The output of the photomultiplier tube was passed through a current to voltage converter and amplifier followed by an R.C. filtering circuit to improve the signal to noise ratio and stored in a 20 MHz Datalab 922 transient recorder. The signal to noise ratio of both the electrochromic and the smaller cytochrome absorption changes were further increased by signal averaging using a Digital Equipment Corporation PDP11-34 computer. This was achieved by adding a number of similar digitally converted signals together. Each signal must be exactly synchronised with the others for the averaging technique to be applied, so that when a number of similar signals are summed the random noise components do not add in reproducible phase and therefore tend to diminish with respect to the signal component. The improvement in the signal component with respect to the noise is proportional to the square root of the number of signal repetitions (Ke, 1972). The digitalised signal was then transferred to the memory of the computer when it and further signals could be manipulated by addition, subtraction, etc. The final signal was plotted out on a Tektronix 4662 plotter. The signals could be written onto and stored on magnetic discs from where they would later be recalled for analysis.

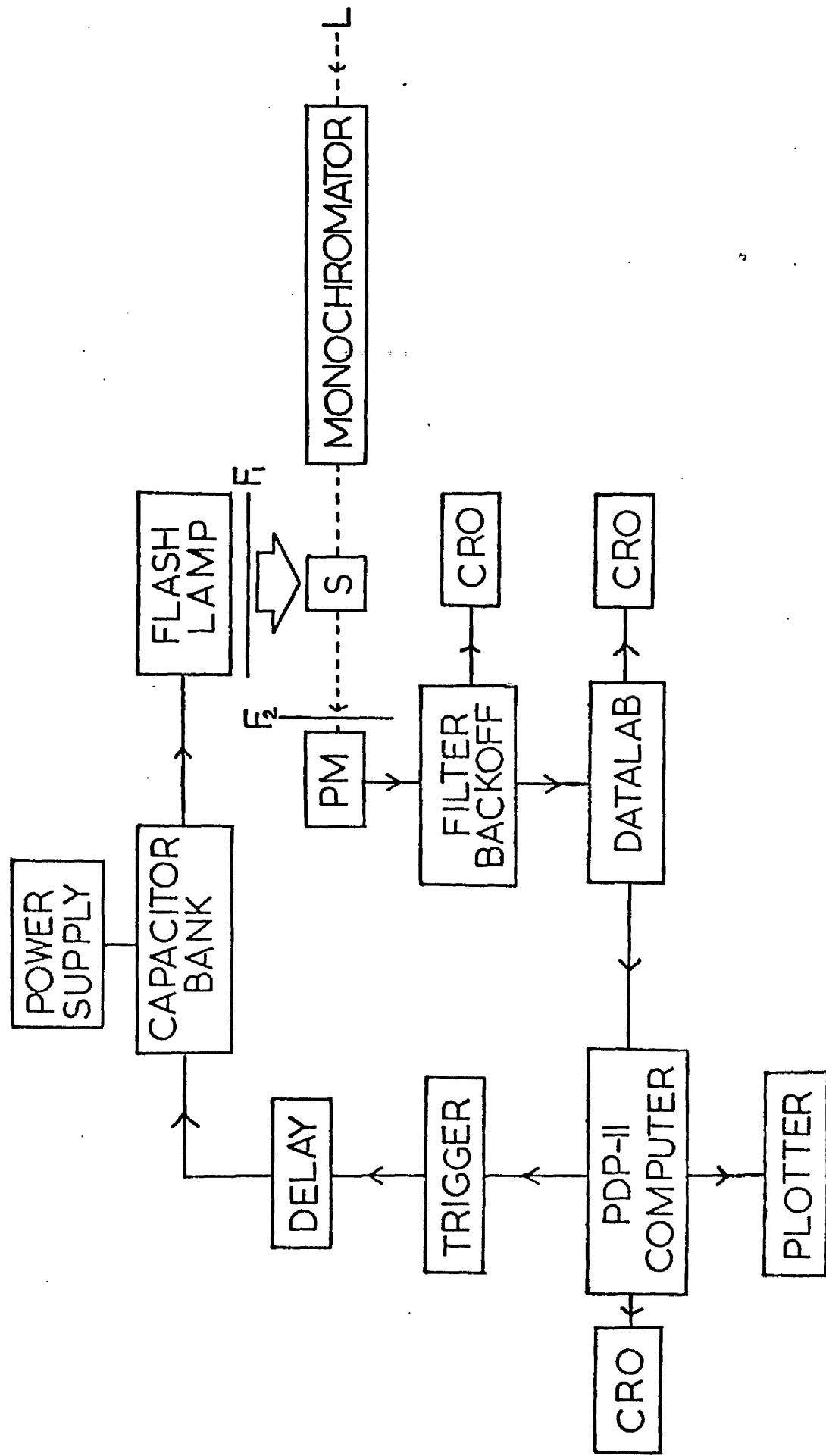


Figure 16. A diagram of the apparatus used to record light-induced absorption changes. L is the measuring beam lamp; S the photosynthetic material in a quartz cuvette; PM the photomultiplier tube; CRO various cathode ray oscilloscopes; F_1 the actinic filters; and F_2 the analysis filters.

Some of the earlier experiments were carried out before the computerised system was installed and consequently the output of the photomultiplier tube was stored on a Tektronix 5115 oscilloscope and the changes recorded by photograph. This prevented averaging and further manipulation of the signal but it was sufficient for those experiments.

Actinic illumination was obtained from a General Radio "Stroboslave" xenon flash lamp, with a duration of approximately 5 μ sec, perpendicular to the axis of the monitoring beam. The frequency and number of actinic flashes were controlled by the computer. Various red filters were placed over the actinic source to optimise the particular cut-off wavelength and intensity appropriate for different experiments e.g. either 2 x Wratten 25 (50% transmission = 600nm), 2 x Wratten 29 (50% transmission = 625nm), 2 x Wratten 92 (50% transmission = 635nm). A complementary set of blue-green filters was placed in front of the photomultiplier tube to eliminate flash-induced artefacts from the scattered actinic light. These varied according to the isolated wavelength of the monitoring beam but generally consisted of 2 x Corning 4-96 (50% transmission = 382-550nm) and either a Barr and Stroud 523nm interference filter or a Wratten 57 (50% transmission = 500-545nm).

The chlorophyll concentration for all absorption change measurements was 30-35 μ g ml⁻¹ except for the heavy metal studies when it was 5 μ g ml⁻¹.

2.7 Measurement of Prompt Chlorophyll Fluorescence

Fluorescence changes were determined at room temperature in a laboratory-built fluorimeter (Figure 17) The sample in a 1cm pathlength, quartz cuvette was excited with a broad-band blue light isolated from a 150W tungsten-halogen bulb by 2 Corning 4-96 and a Balzers Galflex C heat filter giving an intensity of 12 Wm⁻².

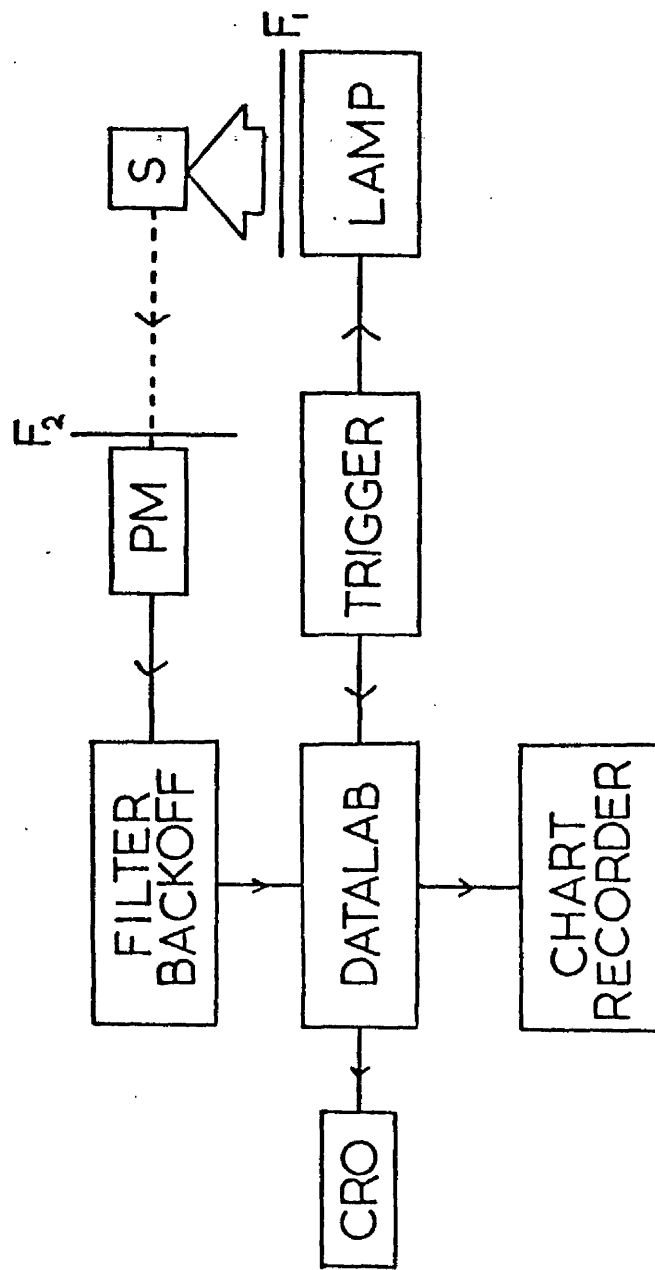


Figure 17. A diagram of the apparatus used to record chlorophyll fluorescence measurements. S is the photosynthetic material in a quartz cuvette; PM the photomultiplier tube; CRO a cathode ray oscilloscope; F_1 the actinic filters and trigger controlled shutter; and F_2 the analysis filters.

The actinic light was directed onto the sample via a solenoid controlled shutter with an opening time of circa 2 msec. The emitted fluorescence was detected at 90° to the path of the exciting light as described for the absorbance changes and stored in a 20MHz Datalab 922 or 200kHz 901 transient recorder. The signal was then plotted on a Servoscribe chart recorder.

The chlorophyll concentration for all fluorescence measurements was between 5 and 10 $\mu\text{g ml}^{-1}$, since reabsorption of the fluorescence was negligible over this range as shown by the linear relationship between fluorescence and chlorophyll concentration (Figure 18).

2.8 Reagents

The reagents used were of analytical grade.

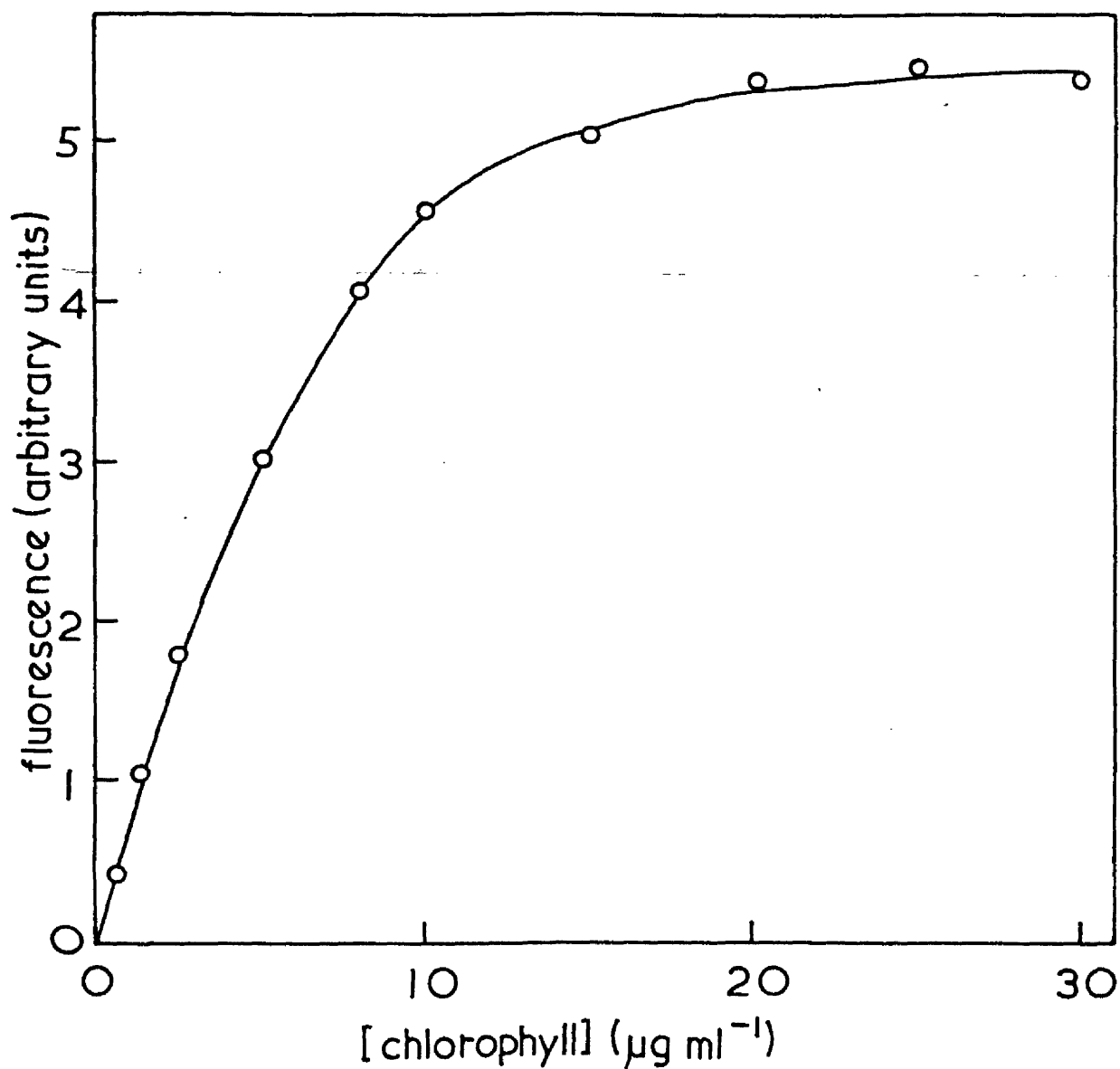


Figure 18. The effect of increasing chlorophyll concentration of the algal sample on the steady state level of fluorescence emission. The figure indicates that reabsorption of fluorescence is negligible for algal chlorophyll concentrations below circa $10 \mu\text{g ml}^{-1}$.

3.1 Results

In order to become accustomed with the techniques of absorption spectroscopy, and to determine which photosynthetic system to work with, it was decided that the first experiments should investigate the effect of varying concentrations of the classical uncoupler, CCCP (see appendix 2 for structural formulae) on the electrochromic absorption change at 515nm. Three different photosynthetic systems were employed;

- (i) Osmotically shocked chloroplasts, isolated from spinach;
- (ii) Chromatophores of the bacterium Rhodospseudomonas capsulata;
- (iii) Intact cells of the green alga Chlorella emersonii.

3.1.1 The effect of CCCP on the Field-Indicating Absorption Change at 515nm

Figure 19 shows the effect of CCCP in the concentration range of 10^{-8}M - 10^{-4}M on the extents and decay half-time of the absorption change at 515nm in spinach chloroplasts and 525nm in chromatophores of Rps. capsulata. Neither system was especially dark adapted nor pre-illuminated so that the redox potentials of their electron carriers would be neither over-reduced or oxidised but would be sitting at an ambient level. In shocked chloroplasts only a fast rise in the extent of the absorption change, within a time shorter than the time resolution of the method, followed by a relatively fast decay ($t_{\frac{1}{2}} = \text{circa } 8.5 \text{ msec}$) is seen. The effect of increasing CCCP concentrations is to decrease the half-time of the decay while the fast phase of increase remains roughly constant until about 10^{-4}M CCCP, when it begins to decrease with further CCCP additions.

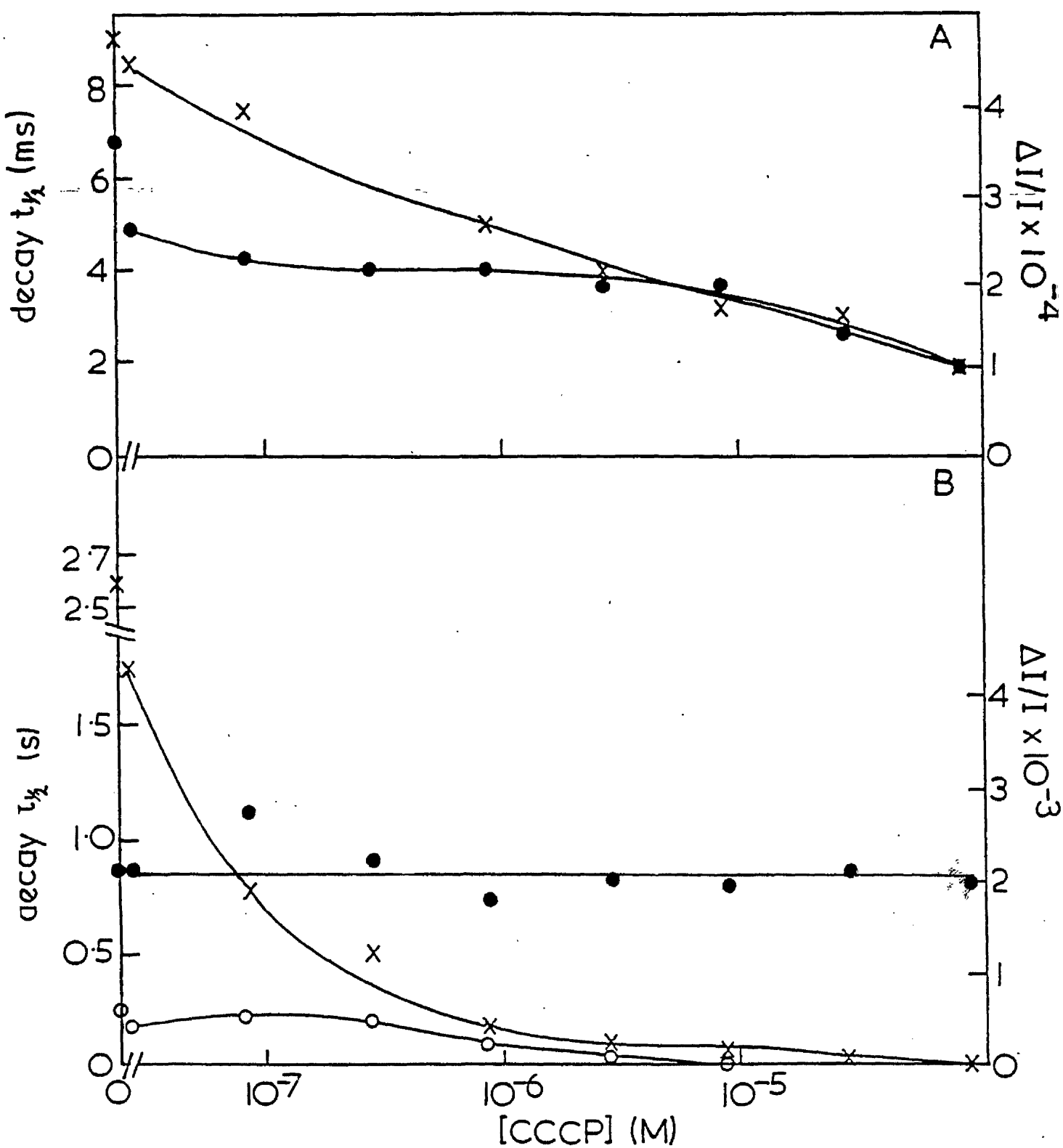


Figure 19. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change; A, at 515nm in osmotically shocked spinach chloroplasts; B, at 525nm in chromatophores of *Rps. capsulata*. No dark adaption or pre-illumination given. (see figure 21 for determination of the extents of the fast and slow phases)

Classic uncouplers like CCCP have the property of increasing the permeability of the thylakoid membrane to protons, and thus abolish phosphorylation without inhibiting electron transport by providing an additional pathway for the dissipation of the proton concentration gradient (see section 1.4). Thus they would be expected to increase the rate of decay of the field-indicating absorption change. The results of Figure 19A are consistent with the explanation that CCCP is acting as a classic uncoupler. It can also be seen that there is no slower phase of absorption increase present in shocked chloroplasts. This is in agreement with reports in the literature (Shahak et al. 1980).

In Rps. capsulata the general trends are similar to those of the shocked chloroplasts with the exception that a slower phase of absorption increase is observed at CCCP concentrations below 10^{-5} but prevented by those above. Again the data suggests the CCCP is acting as a classical uncoupler at concentrations of 10^{-5} M and above (Fleishman and Clayton, 1968; Izawa and Good, 1972).

However, when the same experiment was carried out using the green algae, Chlorella, a different trend was observed (Figure 20A). Instead of the decay half time decreasing with increasing uncoupler concentration, it markedly increased to a maximum at around 10^{-6} M. At approximately the same concentration a slower phase of absorption increase ($t_{\frac{1}{2}} = \text{circa } 3\text{ms}$) appeared which was not present in the non dark-adapted control. The fast phase was not noticeably affected. At concentrations of 10^{-5} M and above, the expected uncoupling effects occurred. Figure 21 shows a typical example of this effect of CCCP on the absorption change in Chlorella at circa 10^{-6} M along with a typical control.

Thus it seemed that in Chlorella only, a large slow phase of absorption increase could be induced by a concentration of CCCP an order of magnitude less than its reported uncoupling concentration.

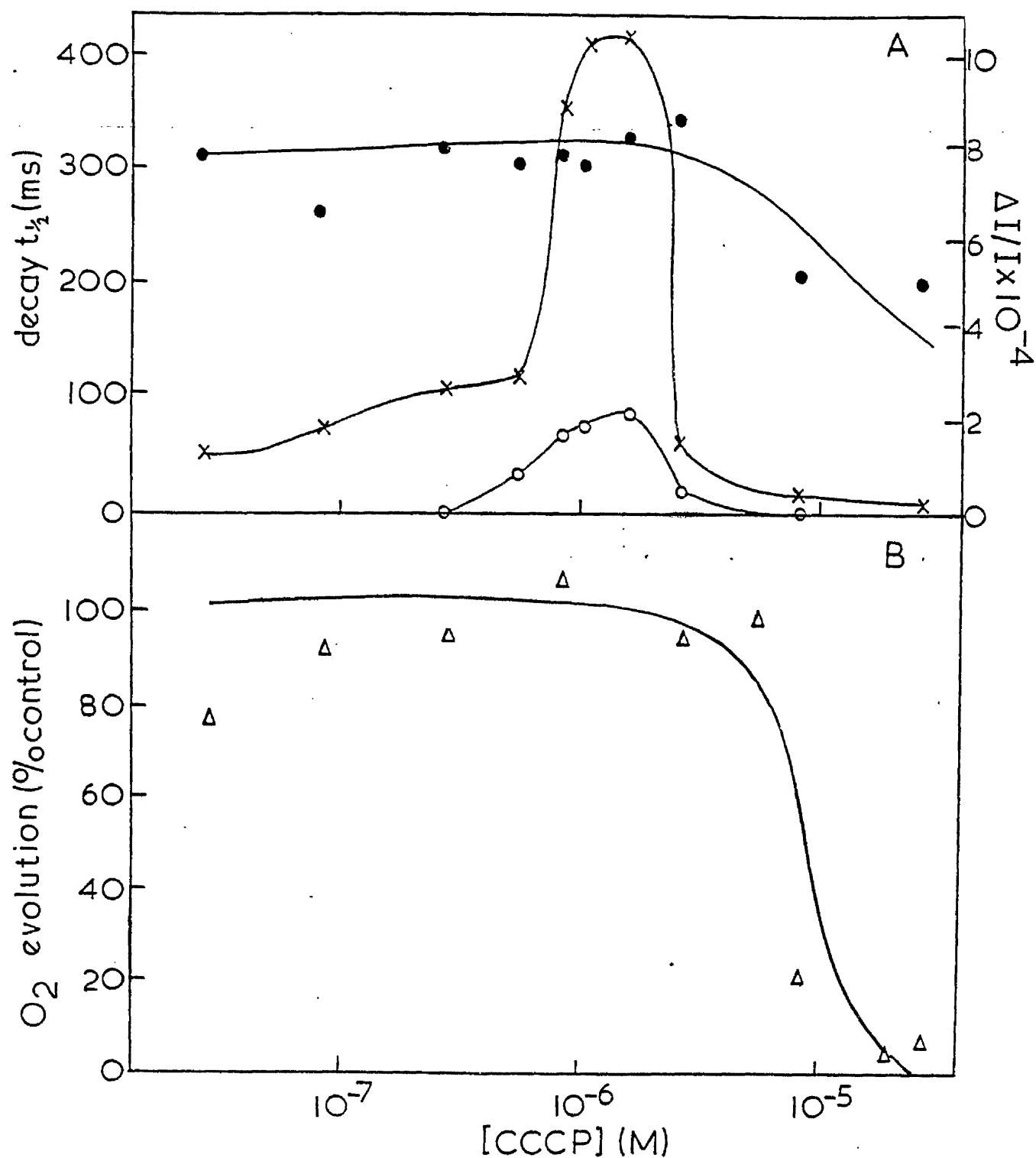


Figure 20. A. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella*. No dark adaption or pre-illumination given. (see figure 21 for determination of the fast and slow phases). B. The effect of increasing concentrations of CCCP on the steady state evolution of oxygen in *Chlorella*. Control rates were from 67-90 $\mu\text{moles } O_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

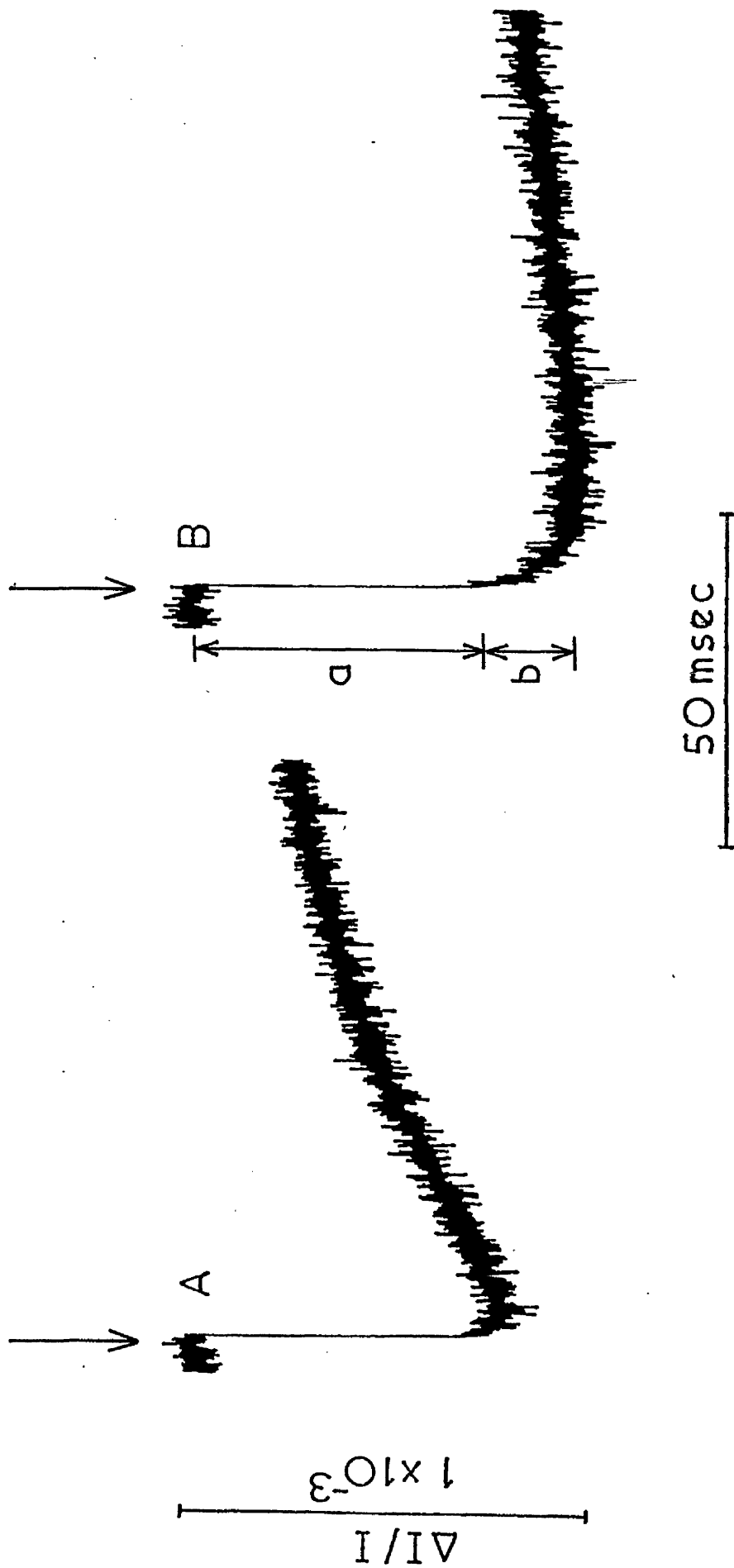


Figure 21. Typical examples of the flash-induced absorption change at 515 nm in Chlorella. A, no additions; B, with the addition of 0.8 μM CCCP. Each trace represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz. The bars, on trace B, marked a and b indicate the extents of the absorption change referred to as the fast phase and slow phase in this thesis. The flash was fired at the point indicated by the arrow.

Amesz and Vredenburg (1966) and Jackson and Crofts (1971) have, however, reported that low concentrations of both CCCP and the similar compound FCCP stimulated the slow phase of the field indicating band shift in the bacterium Rps. sphaeroides but had not discussed or further investigated the phenomena.

To determine whether this stimulatory effect on algae was specific only to CCCP, or a general uncoupler effect, similar experiments were carried out using FCCP and DNP, both phenolic uncouplers thought to act in a similar manner (see appendix 2 for structural formulae). Figures 22A and 23A show that, under similar experimental conditions, the stimulation of the slow phase and decrease of decay half time were also observed using FCCP and DNP at concentrations about an order of magnitude less than their reported uncoupling concentrations (Izawa and Good, 1972).
To determine further whether such an effect was caused by only uncouplers or whether other inhibitors of photosynthesis might show similar results, the effects of adding the inhibitors of cyclic electron flow HQNO and Antimycin A (Izawa and Good, 1972) and the monovalent cation ionophore, Gramicidin (McCarty, 1980) were studied. The experiments proved inconclusive; no effects either stimulatory or inhibitory were observed. As these compounds are known to inhibit photosynthesis in isolated chloroplasts (Junge and Witt, 1968; Slovacek et al. 1979; Izawa and Good, 1972) the results suggested that the molecules were not able to permeate freely into the algal cell chloroplasts as had the phenolic uncouplers.

3.1.2 Light Minus Dark Difference Spectra of the Fast and Slow Phases of Absorption Increase

To ensure that only one type of absorption change was involved during the different phases of absorption increase at 515nm, a light minus dark difference spectrum of both fast and slow phases was derived.

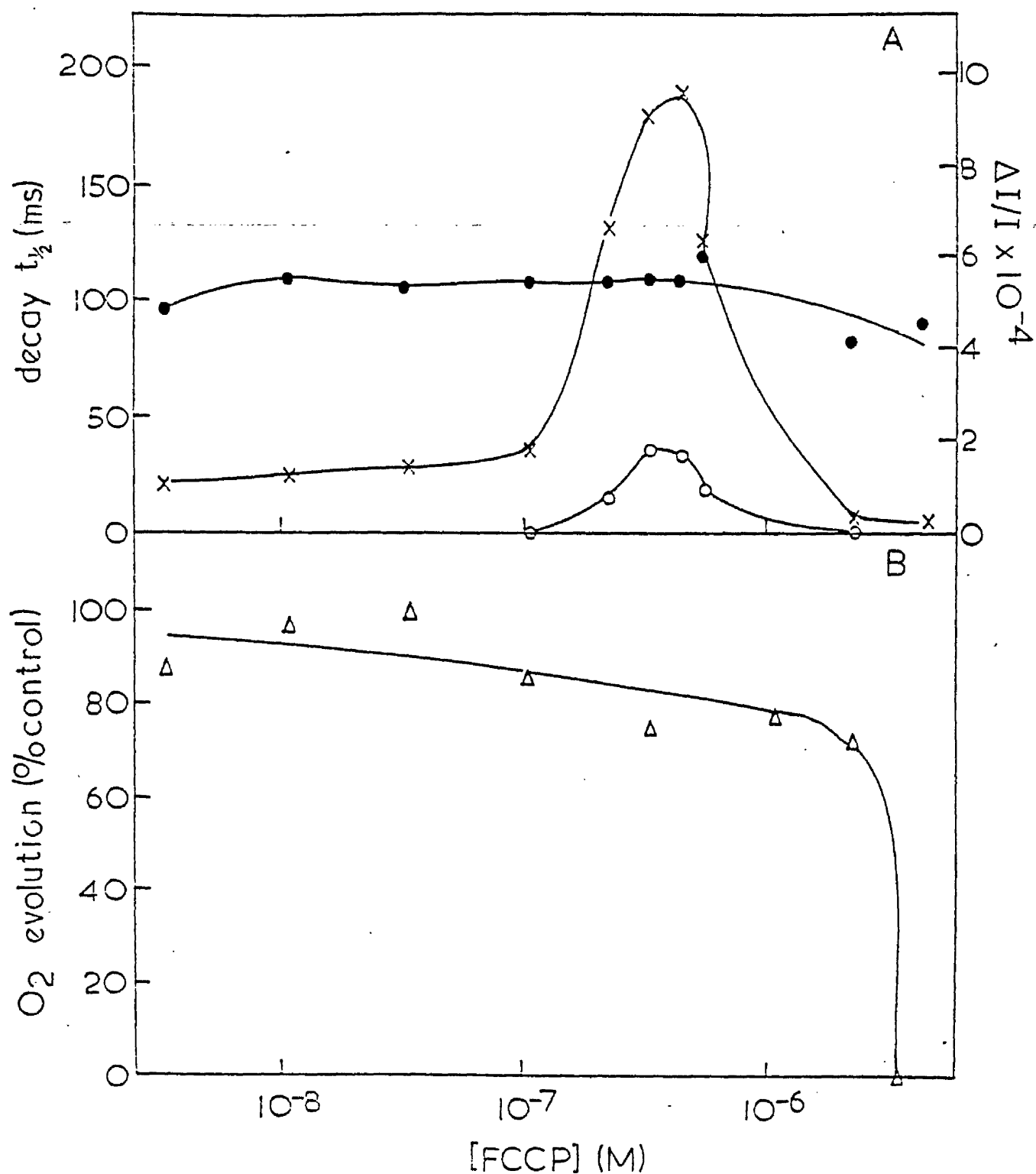


Figure 22. A. The effect of increasing concentrations of FCCP on the fast phase (\bullet), slow phase (o) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella*. No dark adaption or pre-illumination given. B. The effect of increasing concentrations of FCCP on the steady state evolution of oxygen in *Chlorella*.

Control rates were from $81-99 \mu\text{moles } O_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

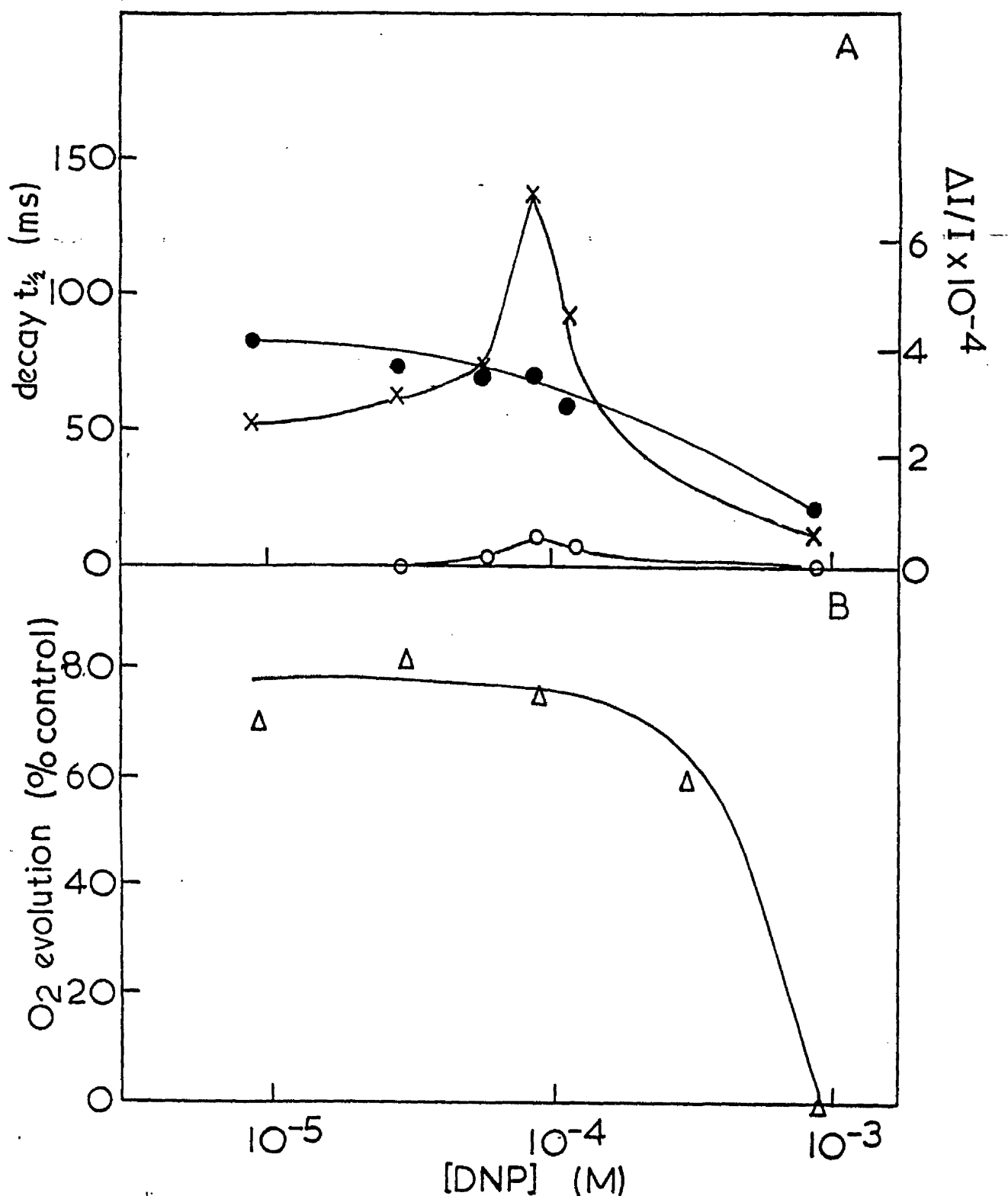


Figure 23. A. The effect of increasing concentrations of DNP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella*. No dark adaption or pre-illumination given.

B. The effect of increasing concentrations of DNP on the steady state evolution of oxygen in *Chlorella*.

Control rates were from $58-67 \mu\text{moles } O_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

The two phases of absorption change were measured at wavelengths between 460nm and 575nm under (a) control conditions and (b) in the presence of 0.8 μ M CCCP to stimulate the slow phase. Figures 24 A and B show that under both types of conditions the spectra for both fast and slow phases are in agreement with those reported for the electrochromic absorption change in algae (Joliot and Delosme, 1974) and in isolated chloroplasts (Slovacek et al. 1979) with a maximum positive peak at 515 - 520nm, a smaller negative peak at 470 - 480nm and an isosbestic point at 490nm. The stimulatory effect of the low concentration of CCCP (0.8 μ M) on the extent of the slow phase is observed across the whole spectrum (B). Above 540nm, on the wing of the 515 peak the absorption changes are no longer purely of the electrochromic shift; other changes are superimposed, notably those at 554nm and 564nm which are assumed to be associated with the α bands of cytochrome f and cytochrome b₅₆₃ (Slovacek et al. 1979; Velthuys, 1979). This difference in the cytochrome region of the spectra is investigated more thoroughly in section 3.1.9.

In order to try to correlate the slow phase with some electrogenic process in the thylakoid membrane the effect of variation of concentration of the phenolic uncouplers was investigated on non-cyclic electron flow and PSII photochemistry in Chlorella.

3.1.3 The Effect of CCCP, FCCP and DNP on Oxygen Evolution in Chlorella

To investigate non-cyclic electron flow as a possible source of the electrogenic step responsible for the slow phase, the effect of varying CCCP concentration on oxygen evolution with CO₂ as the terminal electron acceptor was used (see section 1.5.1 on theory of method). Figures 20B, 22B and 23B respectively show the effect of CCCP, FCCP and DNP on oxygen evolution by Chlorella; in all three cases no stimulation was observed at concentrations which had stimulated the slow phase of the electrochromic absorption change, only a decrease in activity at the uncoupling concentrations of 10⁻⁵M (CCCP), 10⁻⁶M (FCCP), 10⁻³M (DNP).

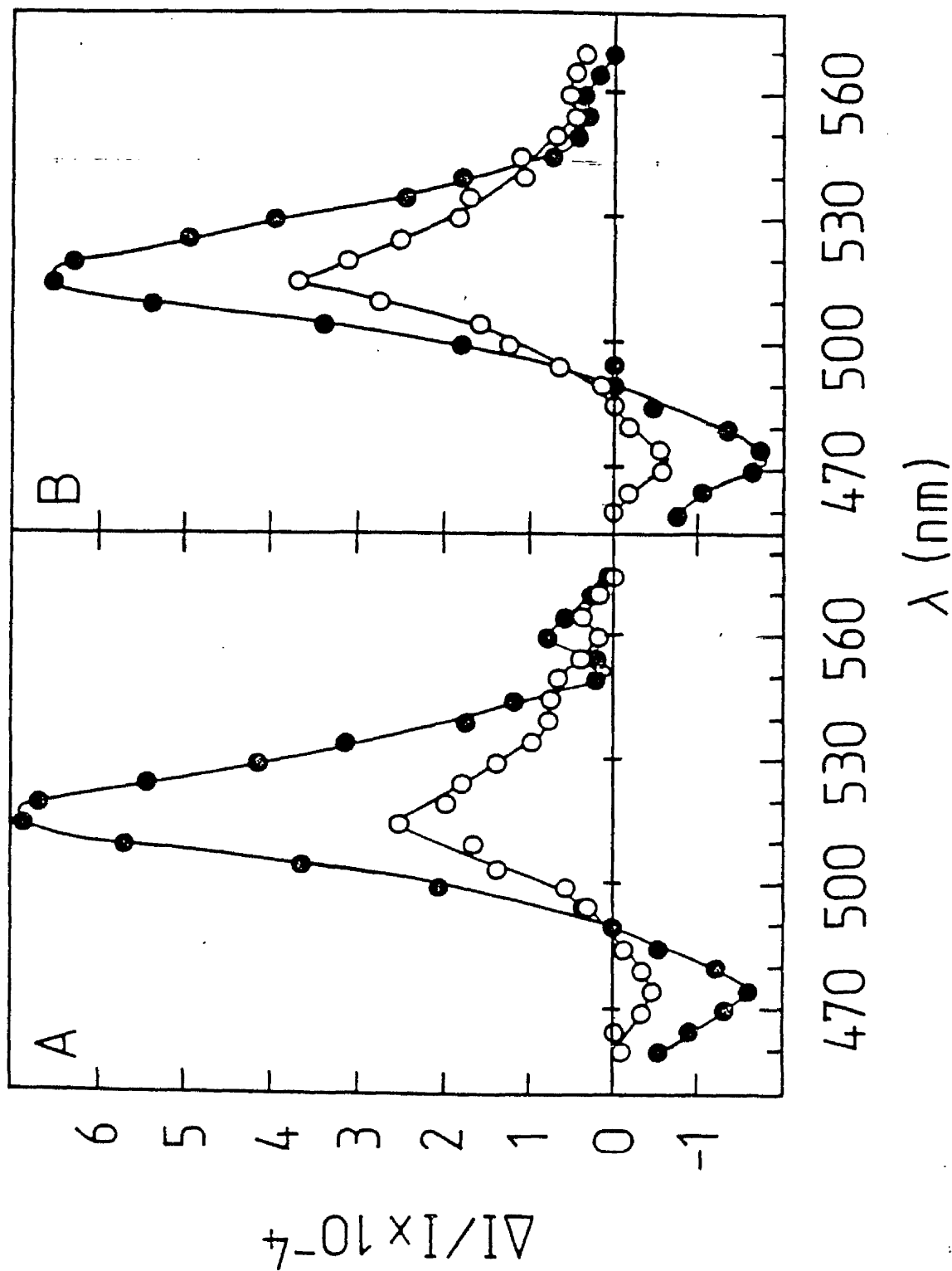


Figure 24. Light minus dark difference spectra of the fast phase (●) and slow phase (○) of the flash-induced absorption change at 515 nm in Chlorella after 5 minutes dark adaptation. A, no additions; B, with the addition of $0.8 \mu\text{M}$ CCCP. Each point represents the accumulated average of between 64 and 250 sweeps at a flash frequency of 0.5 Hz.

In isolated chloroplasts using artificial terminal electron acceptors the effect of uncoupling is to release the energetic constraint of phosphorylation from the electron transport chain by providing an additional pathway for the dissipation of the high energy intermediate. This induces increased rates of electron transport and thus oxygen evolution. However, in intact algal cells, where the terminal electron acceptor is the Calvin cycle of carbon fixation, the inhibition of ATP production by uncoupling would prevent the ATP-driven Calvin cycle. This would lead to a build up of excess NADPH₂ which in turn would lead to a decrease in the rate of non-cyclic electron transport and hence decrease oxygen evolution. The results of Figures 20B, 22B and 23B therefore suggest that the three compounds are acting as classical uncouplers and that the stimulation of the slow phase of the absorption change is not a direct consequence of non-cyclic electron flow as would have been the case if low concentrations of the uncouplers had stimulated the rate of oxygen evolution.

3.1.4 The Effect of CCCP, FCCP and DNP on Chlorophyll Fluorescence

If the stimulating effect of the uncouplers was related to photochemistry, it should be possible to observe changes in the fluorescence induction kinetics (see section 1.5.2) in Chlorella blocked with DCMU. Therefore, the effect of varying uncoupler concentrations was investigated on the three fluorescence parameters; the initial fluorescence level F_0 , the variable fluorescence level F_v , and the relationship $(F_m - F_0)/F_m$ (F_m is the maximum level of fluorescence) which gives an estimate of the quantum efficiency of photochemistry at the PSII reaction centre. Changes in the F_0 level would indicate changes in the rate constants of energy de-excitation (k_h , k_t , k_f) in either (i) pigments not associated with the PSII reaction centre or (ii) in pigments associated with PSII but before the exciton had reached the reaction centre when all the traps are open (section 1.5.2).

Changes in the Fv level might similarly be explained but with the addition that the variable fluorescence is associated with the reduction of the primary electron acceptor Q and thus the closing of traps. Changes in the ratio $(F_m - F_o)/F_m$ would suggest changes in the efficiency of energy trapping by the reaction centre either as changes in the quantum yield of photochemistry (ϕ_{Pmax}) or as changes in the rate constant for radiationless decay at the reaction centre (see section 1.5.2.2) (Van Grondelle and Duysens 1980; Haehnel et al. 1982; Butler and Kitajima, 1975) Figures 25, 26 and 27 show that the respective concentrations of CCCP, FCCP and DNP which stimulated the slow phase of the 515 absorption change had no effect on any of the three parameters of fluorescence studied. These results indicate that the slow phase and its stimulation by low uncoupler concentrations is not related to photosystem II photochemistry and its absorption of light energy. Only concentrations above the reported uncoupling ones had any effect on the three parameters; each compound was found to quench the variable fluorescence level and also the F_o level and the $(F_m - F_o)/F_m$ term to varying extents. This type of quenching has been reported before for compounds such as dinitrobenzene (Etienne et al. 1974) but the high concentration of uncoupler necessary for such effects makes its discussion irrelevant here. However, for a detailed discussion on this subject see the copper discussion (section 4.2).

3.1.5 The Effect of Inhibition of Photosystem II on the Absorption Change at 515nm

To determine whether the slow phase was associated with PSI as had been suggested by the various experiments on fluorescence, the effect of inhibition of PSII with DCMU and NH_2OH (Bennoun, 1970; Joliot and Delosme, 1974) was studied in the presence and absence of $0.8 \mu M$ CCCP.

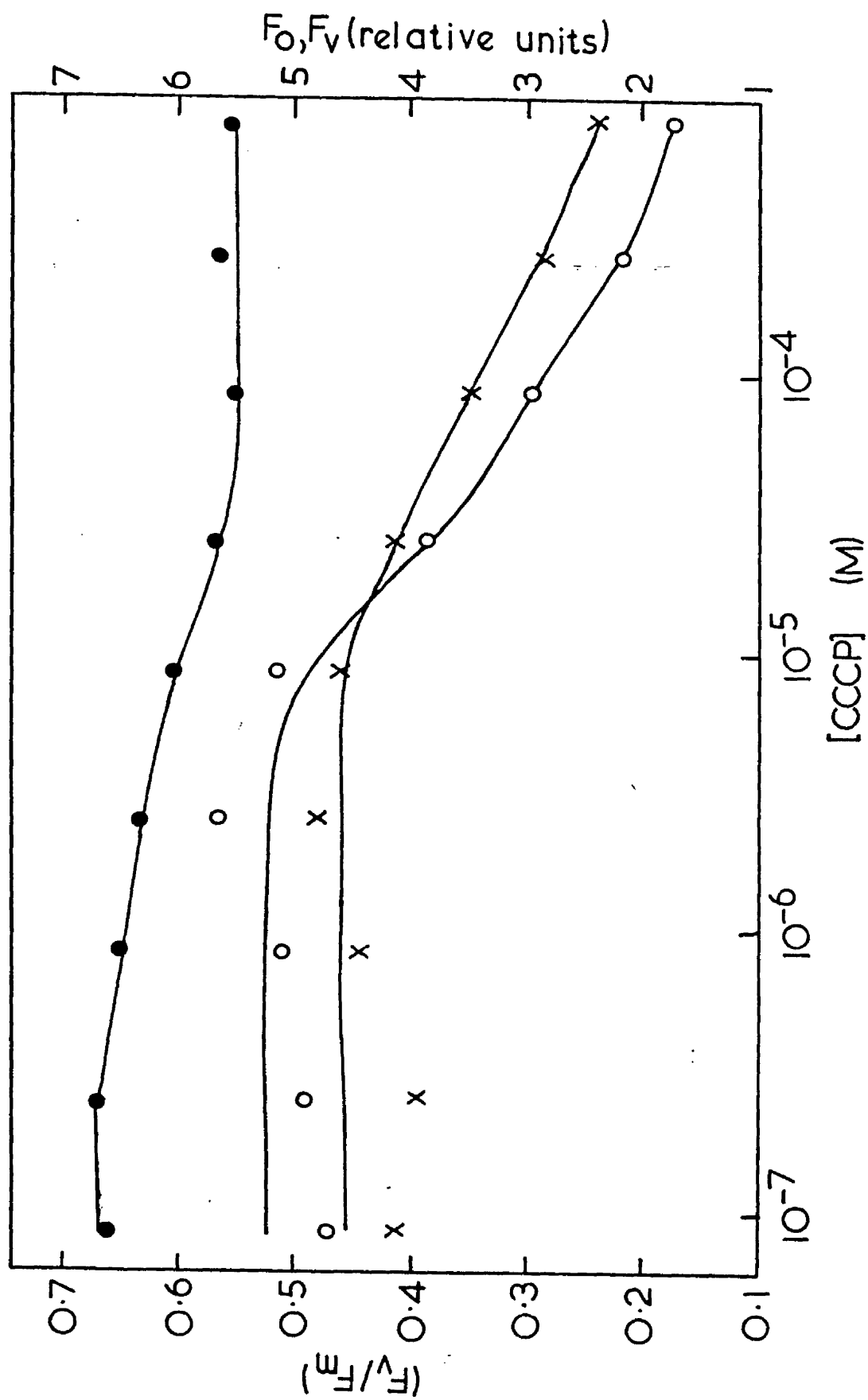


Figure 25. The effect of increasing concentrations of CCCP on the chlorophyll fluorescence induction parameters F_o (\bullet), F_v (\circ) and F_v/F_m (\times) in *Chlorella* after 5 minutes dark adaption in the presence of 10 μM DCMU.

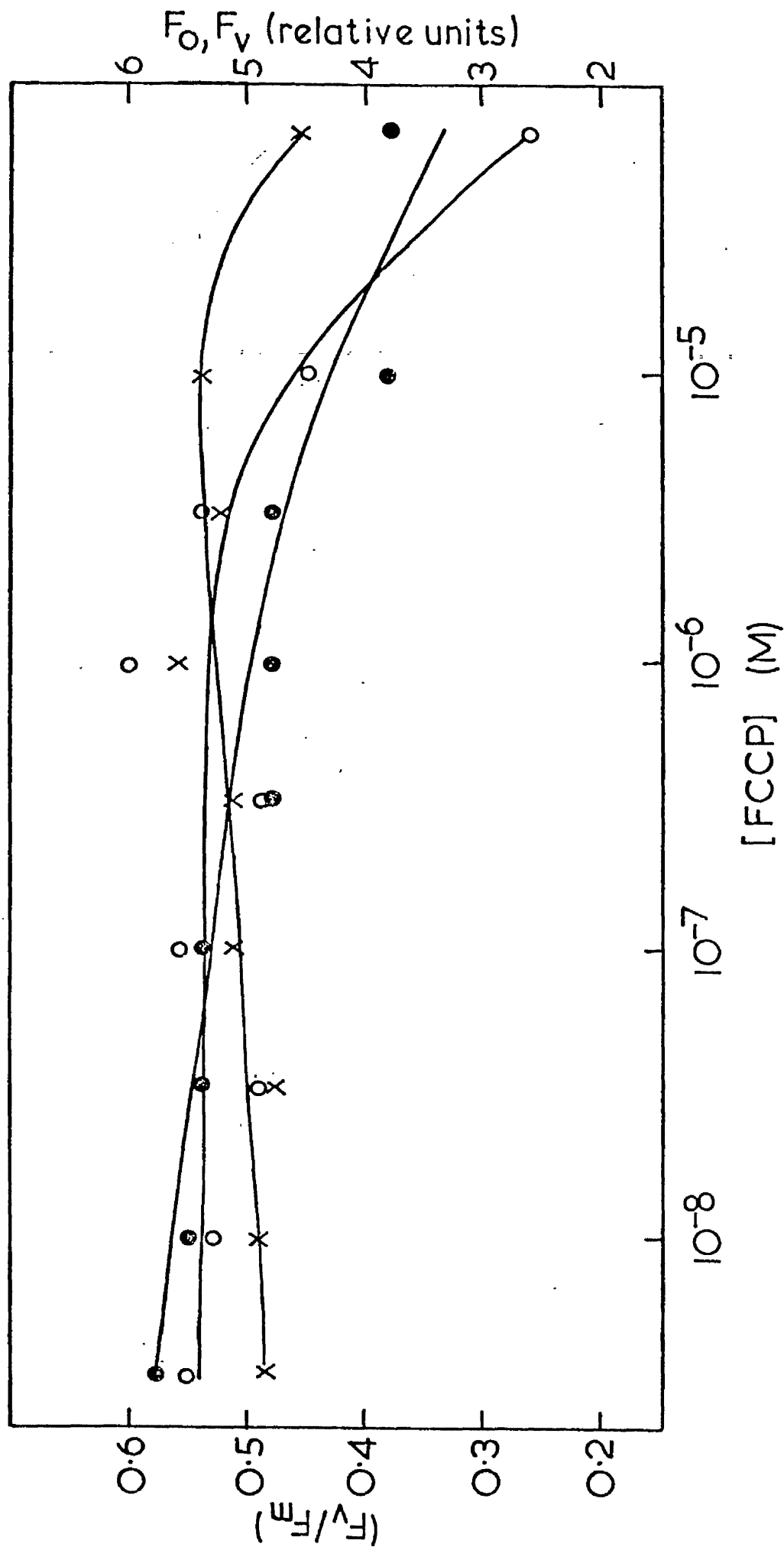


Figure 26. The effect of increasing concentrations of FCCP on the chlorophyll fluorescence induction parameters F_o (●), F_v (○) and F_v/F_m (x) in Chlorella after 5 minutes dark adaption in the presence of $10 \mu\text{M}$ DCMU.

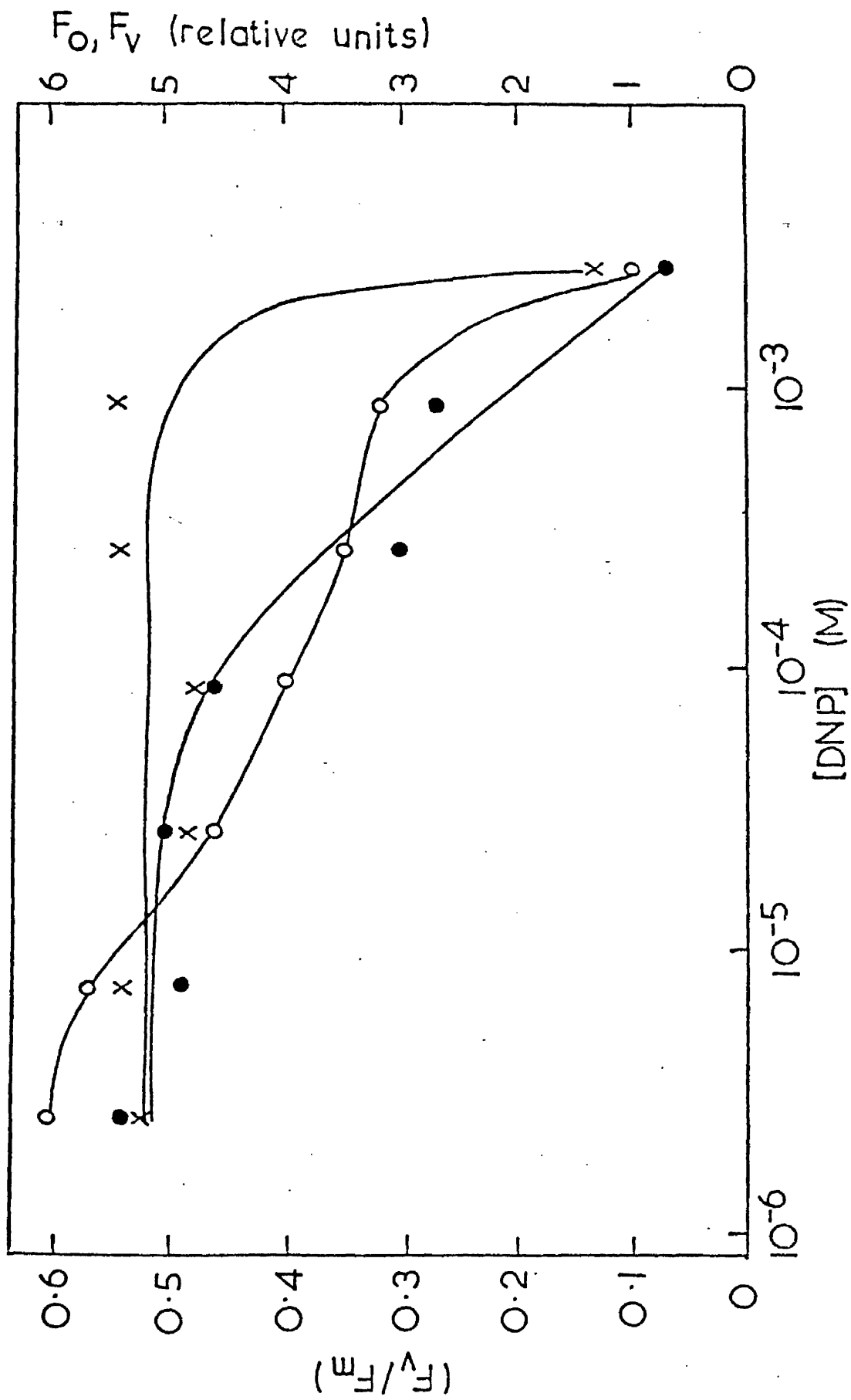


Figure 27. The effect of increasing concentrations of DNP on the chlorophyll fluorescence induction parameters F_o (●), F_v (○) and F_v/F_m (x) in Chlorella after 5 minutes dark adaption in the presence of $10 \mu\text{M}$ DCMU.

When the algae are pre-illuminated in the presence of $10\mu\text{M}$ DCMU and $100\mu\text{M}$ NH_2OH the electron acceptor of PSII, Q, is irreversibly blocked in the reduced form (Bennoun, 1970) and the PSII reaction centre is no longer photoactive. Before the experiment was carried out the amount of inhibition of PSII by the treatment was verified by observing the amount of reduction of the complementary area above the fluorescence induction curve (Malkin and Siderer, 1974). Figure 28 shows that in the presence and absence of $0.8\mu\text{M}$ CCCP the complementary area above the fluorescence curve is inhibited in a similar fashion by about 60% (actually 55% and 65% respectively). This suggests that the treatment is not completely inhibiting PSII. However this figure is misleading. ~~(X)~~ The fluorescence induction rises in two kinetic phases, one fast and the other much slower (see Figure 28) (Etienne, 1974a). It has been reported that even at extremely high concentrations of DCMU the slow component of the induction still persists. Recently a number of workers (e.g. Horton and Croze, 1979) have reported two electron acceptors of PSII at different redox potentials. Horton and Croze (1979) propose that the reduction of the lower potential component corresponds to the fast phase of the induction curve, whilst the slower reduction of the higher potential component accounts for the slow tail phase. The inhibition treatment appeared to reduce the fast rising component almost completely but had little effect on the slow secondary rise. Therefore, instead of the reduction of area above the curve being almost 100%, 40% above the slow rise remained. It was assumed therefore that most of the PSII reaction centres had been deactivated whilst the non-inhibition of the slow tail phase remained unexplained.

Figure 29 shows the effect of blocking PSII by pre-illumination for 10 seconds with $10\mu\text{M}$ DCMU and $100\mu\text{M}$ NH_2OH followed by 5 minutes dark incubation, on the 515 absorption change.

Figure 28. Inhibition of PSII in Chlorella by 10 μM DCMU and 100 μM NH_2OH followed by 10 s pre illumination (white light giving an intensity of 20Wm^{-2}) as shown by the reduction of the area above the fluorescence induction curve after 5 minutes dark adaption. A, with the addition of 10 μM DCMU; B, with the addition of 10 μM DCMU and 100 μM NH_2OH ; C, as A but with the addition of 0.8 μM CCCP; D, as B but with the addition of 0.8 μM CCCP.

The area above the curve was measured from the time at which the exciting light was switched on to the end of the measured transient.

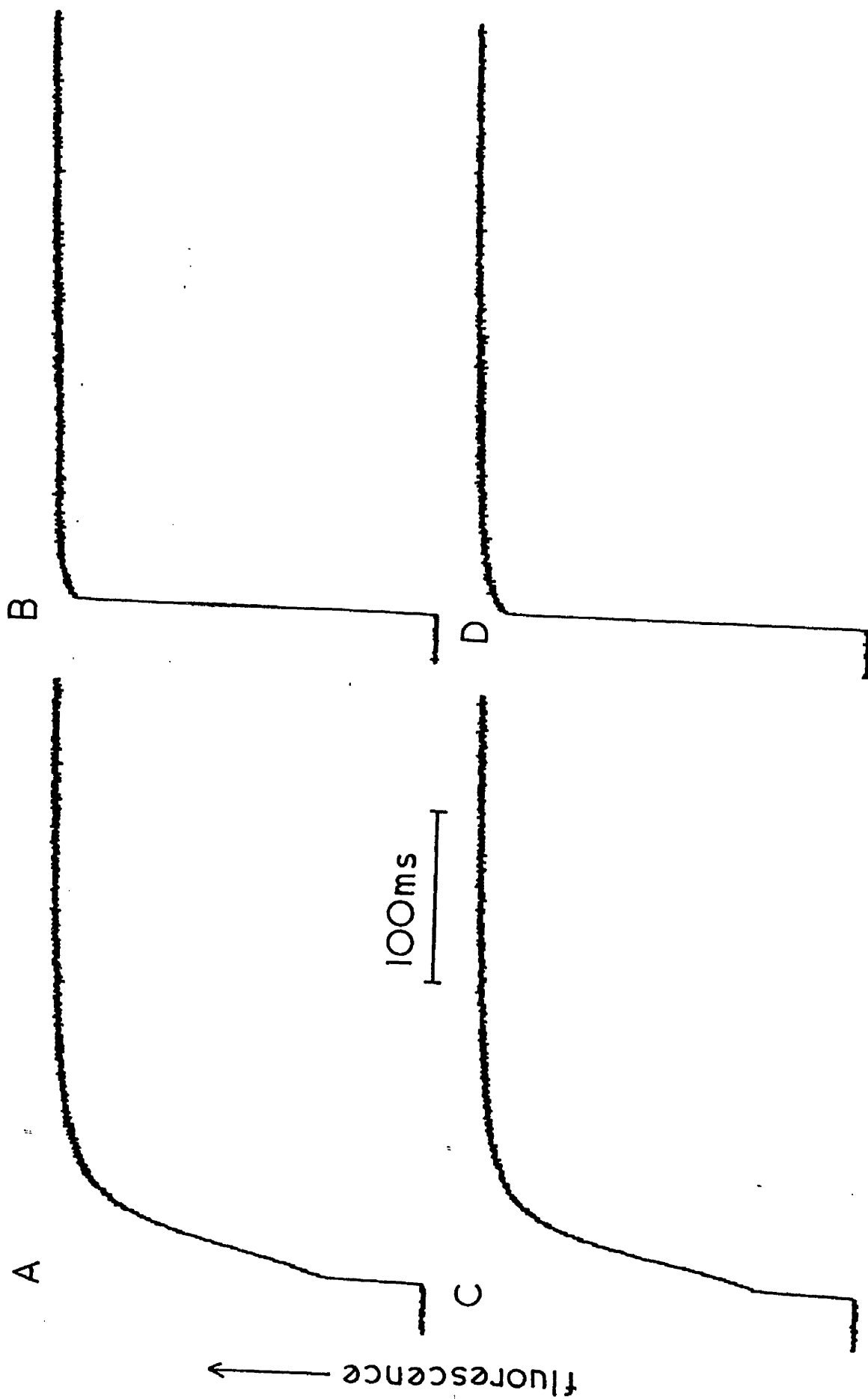
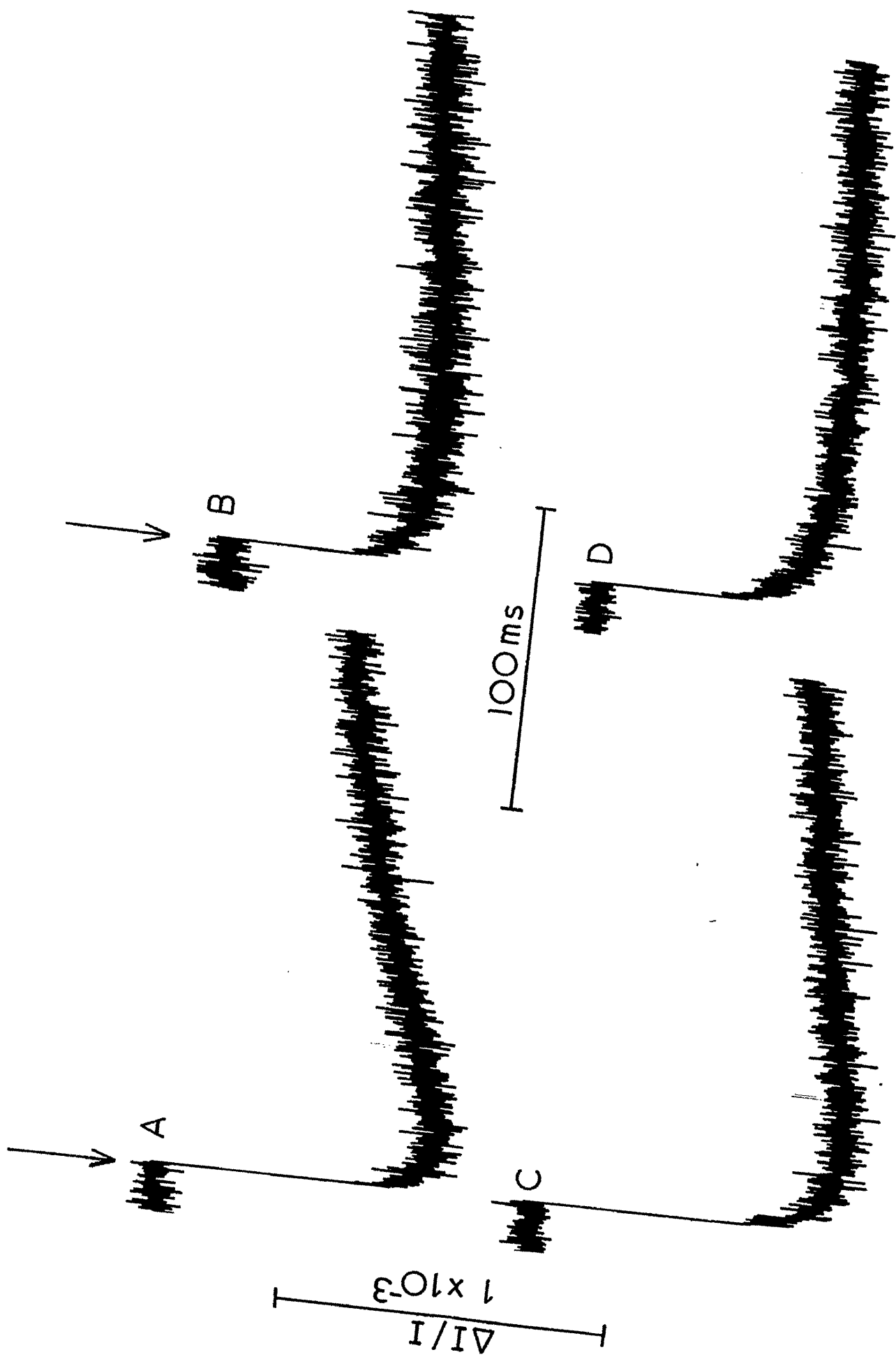


Figure 29. Typical examples of the flash-induced absorption change at 515 nm in Chlorella with PSII blocked by the addition of 10 μM DCMU and 100 μM NH_2OH followed by 10 s pre-illumination (white light giving an intensity of 20 Wm^{-2}) after 5 minutes dark adaption. A, no additions; B, with the addition of 10 μM DCMU and 100 μM NH_2OH ; C, as A but with the addition of 0.8 μM CCCP; D, as B but with the addition of 0.8 μM CCCP. Each trace represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz. The flash was fired at the point indicated by the arrow.



Furthermore $0.8\mu\text{M}$ CCCP was added in traces C and D to stimulate the extent of the slow phase. The data is summarised in Table 1.

Table 1

Percentage Inhibition of the Fast and Slow Phases of Absorption Increase at 515nm by $10\mu\text{M}$ DCMU and $100\mu\text{M}$ NH_2OH followed by 10 seconds pre-illumination by White Light of Intensity circa 20 Wm^{-2}

<u>Sample</u>	<u>Percentage Inhibition</u>	
	<u>Fast Phase</u>	<u>Slow Phase</u>
No addition	37.5	0
+ $0.8\mu\text{M}$ CCCP	33.0	0

It shows that under both types of conditions inhibition of PSII reduced the fast phase of absorption increase by approximately 35% whereas the slow phase was relatively unaffected. This suggests that PSI contributes up to 65% of the fast phase and most or all of the slow phase while PSII only contributes up to 35% of the fast phase. This agrees with the results of Joliot and Delosme (1974) in Chlorella but is in contrast with the results of Schliephake et al. (1968) in isolated chloroplasts where the contribution of each photosystem was found to be approximately equal. Secondly the inhibition of PSII had no effect on the slow phase either when stimulated with $0.8\mu\text{M}$ CCCP or under control conditions. This would indicate that in agreement with the fluorescence data, the stimulatory effect of low concentrations of uncoupler is not directly associated with PSII.

3.1.6 The Effect of Dark Adaption on the Slow Phase of Absorption Increase at 515nm

Joliot and Delosme (1974) and Velthuys (1978) reported that the slow phase was dependent on the amount of pre-illumination of algae or chloroplasts before flash measurements were taken. Thus in order to ascertain whether the stimulatory effect of low uncoupling concentrations was related to light exposure, the effect of varying dark adaption after a standard illumination period was investigated. The algae were first pre-illuminated by a strong red source (2 Schott RG 620 filters giving an intensity of 60 Wm^{-2}) and then subjected to varying dark times before the flash-induced absorption change was measured. Figure 30 shows the results of varying dark adaption periods on the extent of the slow phase in the presence and absence of $0.8 \mu\text{M}$ CCCP. In the control the slow phase is absent until about 7.5 minutes of dark adaption have elapsed. The slow phase then increases with increased dark time until a maximal value is reached. In the presence of $0.8 \mu\text{M}$ CCCP the slow phase appeared after a much shorter dark adaption period (about 3 minutes) and the maximal value was reached after a much shorter dark adaption than in the control. Furthermore a greater maximum level was reached in the presence of $0.8 \mu\text{M}$ CCCP as might be expected from the previous data (Figure 20A). In both cases the fast phase remained unaltered. This data obtained with Chlorella suggests firstly that $0.8 \mu\text{M}$ CCCP has the property of accelerating the dark adaption process which itself brings about the slow phase. Secondly the data also suggests that steady state illumination inhibits the slow phase of absorption increase, i.e. during continuous turnover and under light saturating conditions the process responsible for the slow phase is not active. Finally the fast phase does not seem to be dependent on pre-illumination or dark adaption conditions. Velthuys (1978) however, finds the opposite result in isolated chloroplasts and that pre-illumination is a prerequisite for the appearance of the slow phase in that system. The effect of pre-illumination is therefore investigated in isolated chloroplasts in later sections, (3.1.11.2) for intact chloroplasts and 3.1.11.4 for osmotically shocked chloroplasts.

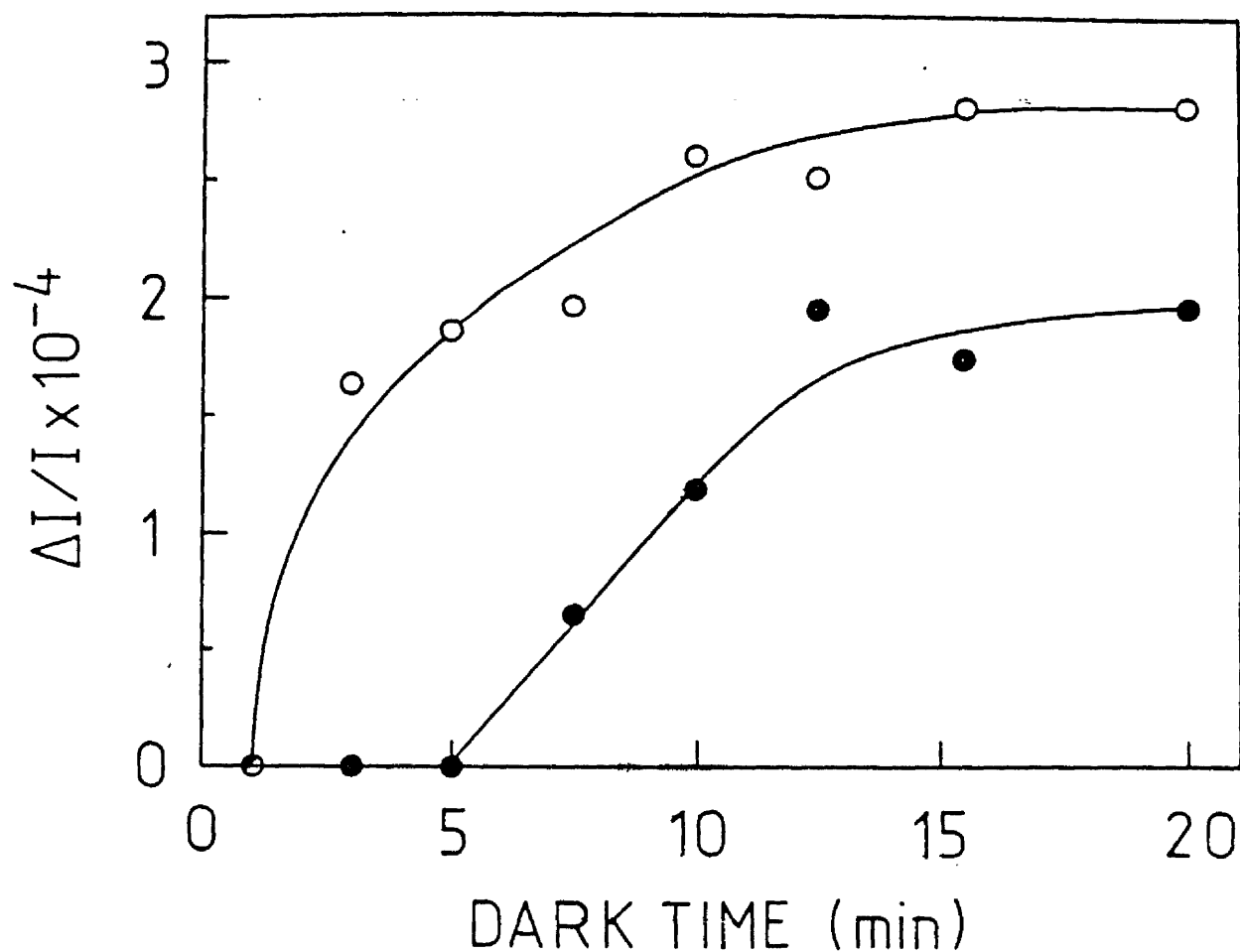


Figure 30. Dependence of the slow phase of the flash-induced absorption change at 515 nm in Chlorella on the length of dark adaption after 15 seconds pre-illumination with a red actinic source (2 Schott RG 620 red filters giving an intensity of 60 Wm^{-2}). ●, no additions; o, with the addition of $0.8 \mu\text{M}$ CCCP.

3.1.7 The Effect of a Series of Flashes on the Absorption Change at 515nm

To investigate further the dependence of the slow phase of absorption increase at 515nm on the light treatment conditions, the absorption changes induced by a series of flashes were studied both in the presence and absence of 0.8 μ M CCCP to stimulate the slow phase. In neither case were the algae pre-illuminated or dark adapted to any great extent beforehand as in the experiments of section 3.1.1.1. Figure 31 show the results of giving nine actinic flashes, at a flash frequency of 5 Hz, to Chlorella under conditions of (A) control and (B) maximum stimulation of the slow phase by addition of 0.8 μ M CCCP. Figure 31 A is on a two times more sensitive scale than B. In the case of the control very little variation occurs from one flash to another. The frequency of flashing prevents each of the flash-induced transients from decaying completely and thus a small threshold is set up. In the case of the sample incubated with 0.8 μ M CCCP a different pattern is seen. Firstly the rate of decay of each flash-induced transient is decreased, as expected from Figure 20A, the rate being slowest on the first flash and increasing with each flash. As in the control the transients do not decay completely between each flash and so each flash-induced transient accumulates until a relatively high threshold is reached on about the seventh flash when no further increase is seen. The slow phase follows a complementary sequence, being maximal on the first flash and decreasing until it disappears on the seventh flash. The accumulating effect of the first six flashes and the increasing rate of decay may be explained by the flash-induced field across the thylakoid membrane not decaying fast enough between each flash for it to be dissipated and thus it accumulates until a point is reached when the charge separated across the membrane is equal to the field decaying between each flash and a threshold is reached.

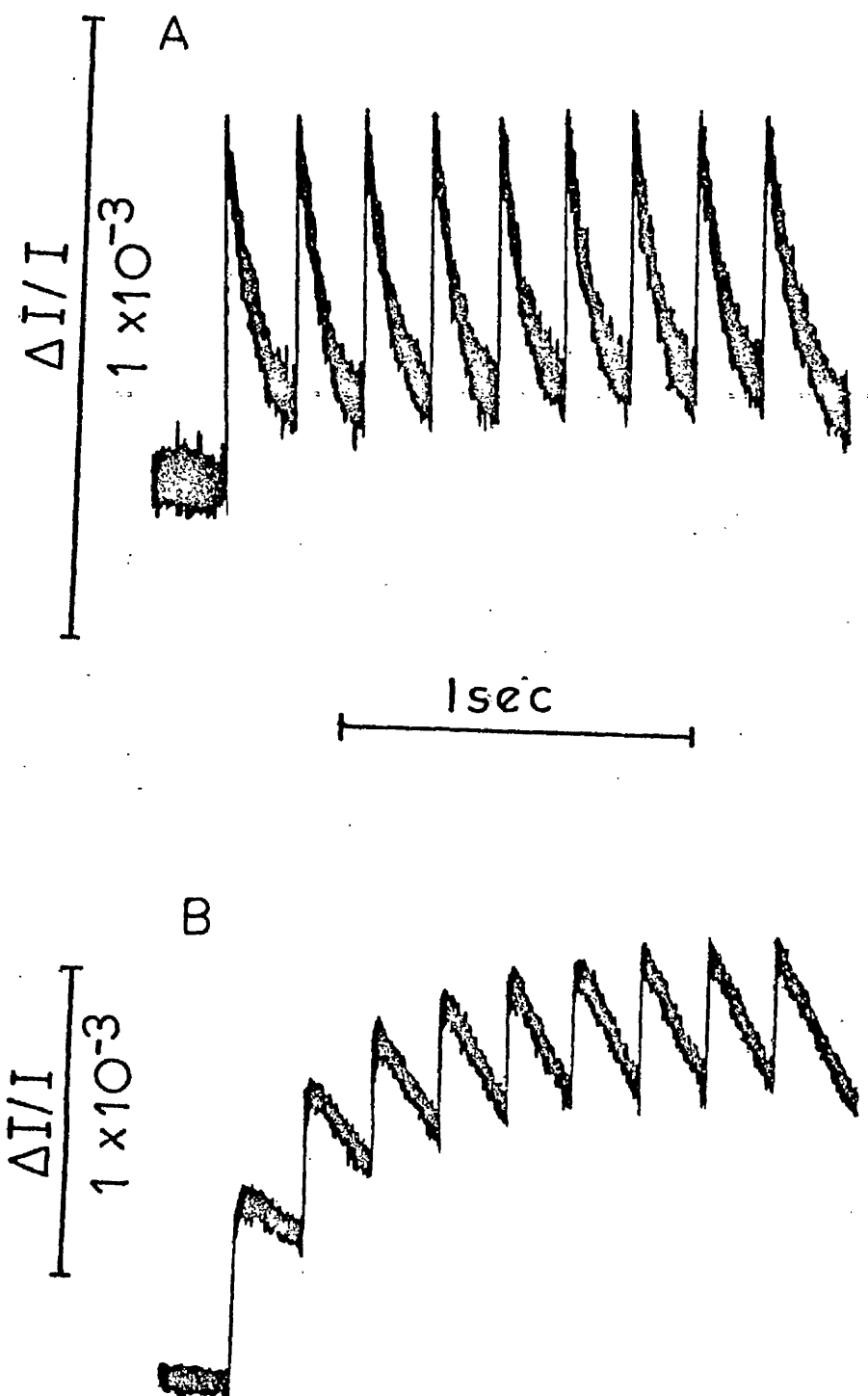


Figure 31. Time course of the absorption change at 515 nm induced by a series of flashes in *Chlorella*. No dark adaption or pre illumination given. A, no additions; B, with the addition of 0.8 μM CCCP. The time course in A was obtained on a two times more sensitive scale than B.

The decreasing extent of the slow phase may be due to a similar mechanism to that which induces pre-illumination to inhibit the slow phase. Firstly a light-induced redoxpoising of electron transport components to a state not capable of supporting the slow electrogenic step may be induced. Secondly the build up of a large electrochemical gradient across the membrane would increase the energy necessary to draw electrons across the membrane in the same direction, which results in the slow phase. Thus a point is reached when the free energy liberated by the reaction coupled to the electrogenic step is no longer sufficient to draw an electron across the membrane against the transmembrane electrochemical gradient. Therefore, the larger the electrochemical gradient the larger the inhibition of the slow phase. Bourges-Bocquet (1981) has presented evidence for this hypothesis. She suggests that a large electric field across the membrane inhibits the slow phase due to the free energy available for driving electrons across the membrane being insufficient to overcome the back pressure of an increasing electrochemical gradient if the reaction which transfers electrons across the membrane is coupled to inward proton pumping. Thus an increasing electrochemical gradient prevents the inward pumping of protons which results in a decreasing rate of the electron-proton coupled step. Under normal conditions the electrochemical gradient would not reach such high levels, since proton efflux via phosphorylation (or increased proton permeability under uncoupled conditions) would decrease the electrochemical gradient. However, under the conditions of Figure 31(B) where the decay of the transmembrane field is markedly slowed the electrochemical gradient might reach abnormally high transient levels. A third alternative explanation may be that under conditions of normal physiological decay rates (Figure 31A) the slow phase is hidden by the rapid decay of the 515 absorption change.

One further point to be drawn from these results is that there is no periodicity of four, which is seen in flash-induced oxygen evolution transients (see section 1.5.1.1). Some authors have reported oscillations in the absorption change although they are generally of a binary oscillation rather than with a period of four as seen with oxygen yields (Bourges-Bocquet, 1980; Velthuys, 1980b). Velthuys (1980b) correlated this binary oscillation with proton release inside the thylakoid and interpreted the oscillation as evidence for an extra site of proton translocation being responsible for the slow phase. Bourges-Bocquet (1980) on the other hand found that the oscillations were sensitive to DCMU suggesting that the electrons arriving at the reductant necessary for the slow phase (see section 1.5.3.5.1) were originating from the secondary acceptor of PSII, B or R.

3.1.8 The Effect of Plastocyanin Inhibitors on the Absorption Change at 515nm and Oxygen Evolution

As the previous results had suggested that the slow phase of absorption increase at 515nm was associated with PSI and probably not PSII it was of interest to discover whether blocking one of the primary electron donors of PSI, plastocyanin (see section 1.3.3.3) had any effect on the slow phase. A number of compounds have been reported to react with plastocyanin and block electron transport from cytochrome f to the PSI reaction centre (Trebst, 1980). The two compounds chosen were KCN (Ouitrakul and Izawa, 1973) and HgCl_2 (Kimimura and Katoh, 1972). Figure 32 shows the effect of KCN on the absorption change at 515nm in Chlorella. Surprisingly, the results indicate that, like the uncouplers, CCCP, FCCP and DNP, KCN at concentrations greater than 10^{-5}M stimulates the extent of the slow phase and markedly decrease the rate of decay of the absorption change. Like the uncouplers the fast phase was largely unaffected until high concentrations ($> 10^{-2}\text{M}$) when it decreased slightly. Unlike the uncoupler data however increasing the KCN concentration above that which stimulates the extent of the slow phase did not significantly decrease its extent or markedly increase the rate of decay, even at concentrations as high as $3 \times 10^{-2}\text{M}$.

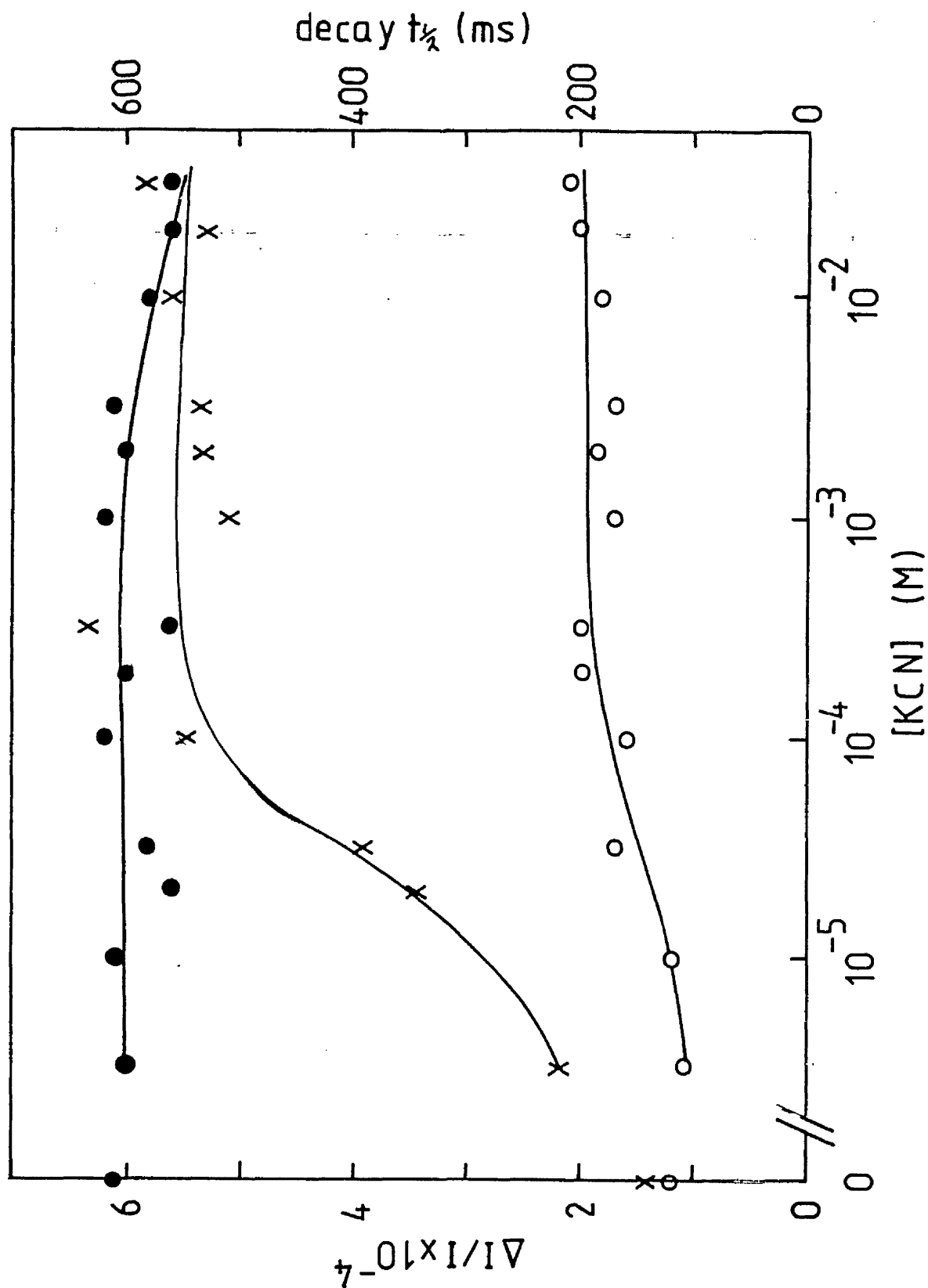


Figure 32. The effect of increasing concentrations of KCN on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella* after 5 minutes dark adaption. Each point represents the accumulated average of 32 sweeps at a flash frequency of 0.5 Hz.

Figure 33 shows the effect of KCN concentration on oxygen evolution in Chlorella with CO₂ as the terminal electron acceptor. The experiment was carried out to determine the point of inhibition of photosynthesis in Chlorella by KCN and in a similar manner to the uncoupler data determine whether the concentrations of KCN which stimulate the slow phase of absorption increase at 515nm had any effect on the overall photosynthetic rate. The results show a gradual decrease in oxygen evolution with increasing KCN concentration from $10^{-5}M$ - $10^{-2}M$ which is the concentration reported to inhibit chloroplast electron transport (Trebst, 1980). Interestingly, the range of concentrations over which the stimulation of the slow phase of absorption increase at 515nm takes place (50% stimulation = $3.0 \times 10^{-5}M$) is the same as that which gradually decreases oxygen evolution (50% stimulation = $2.4 \times 10^{-5}M$).

To discover whether other plastocyanin inhibitors displayed similar properties as KCN, HgCl₂, which is thought to block electron transport at the same step was used (Trebst, 1980). It is noteworthy here to mention that the effect of HgCl₂ on a number of photosynthetic parameters is studied in more detail in the section on heavy metals (section 4.2). As slightly different conditions of resuspending media, chlorophyll concentration etc were employed in that section, the effect of HgCl₂ on the absorption change at 515nm in Chlorella was carried out under the conditions employed in the other experiments of this section, such as those of uncoupler and KCN concentration. Figure 34 shows the results of varying HgCl₂ concentration on the absorption change at 515nm. Again HgCl₂ is also responsible for stimulating the slow phase although over a much narrower concentration range than KCN, with a maximum effect at about $10^{-4}M$. Like the other stimulatory treatments HgCl₂ induced the rate of decay to decrease markedly at the stimulatory concentration. As in the case of the uncouplers, but unlike KCN, increasing the concentration above $10^{-4}M$ markedly inhibits the slow phase, increases the rate of decay and also markedly reduces the fast phase.

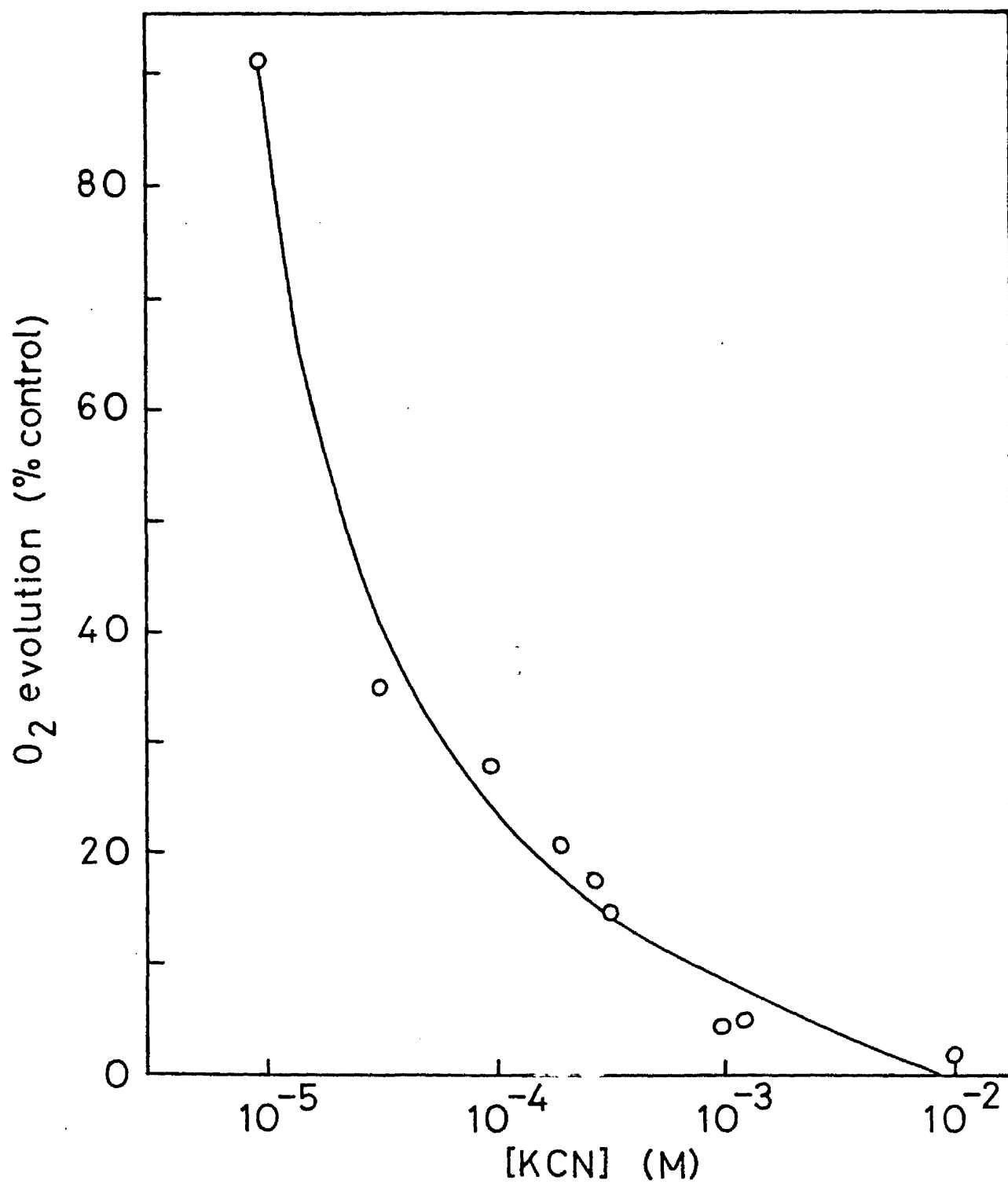


Figure 33. The effect of increasing concentrations of KCN on the steady state evolution of oxygen in Chlorella.

Control rates were from 133-177 $\mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

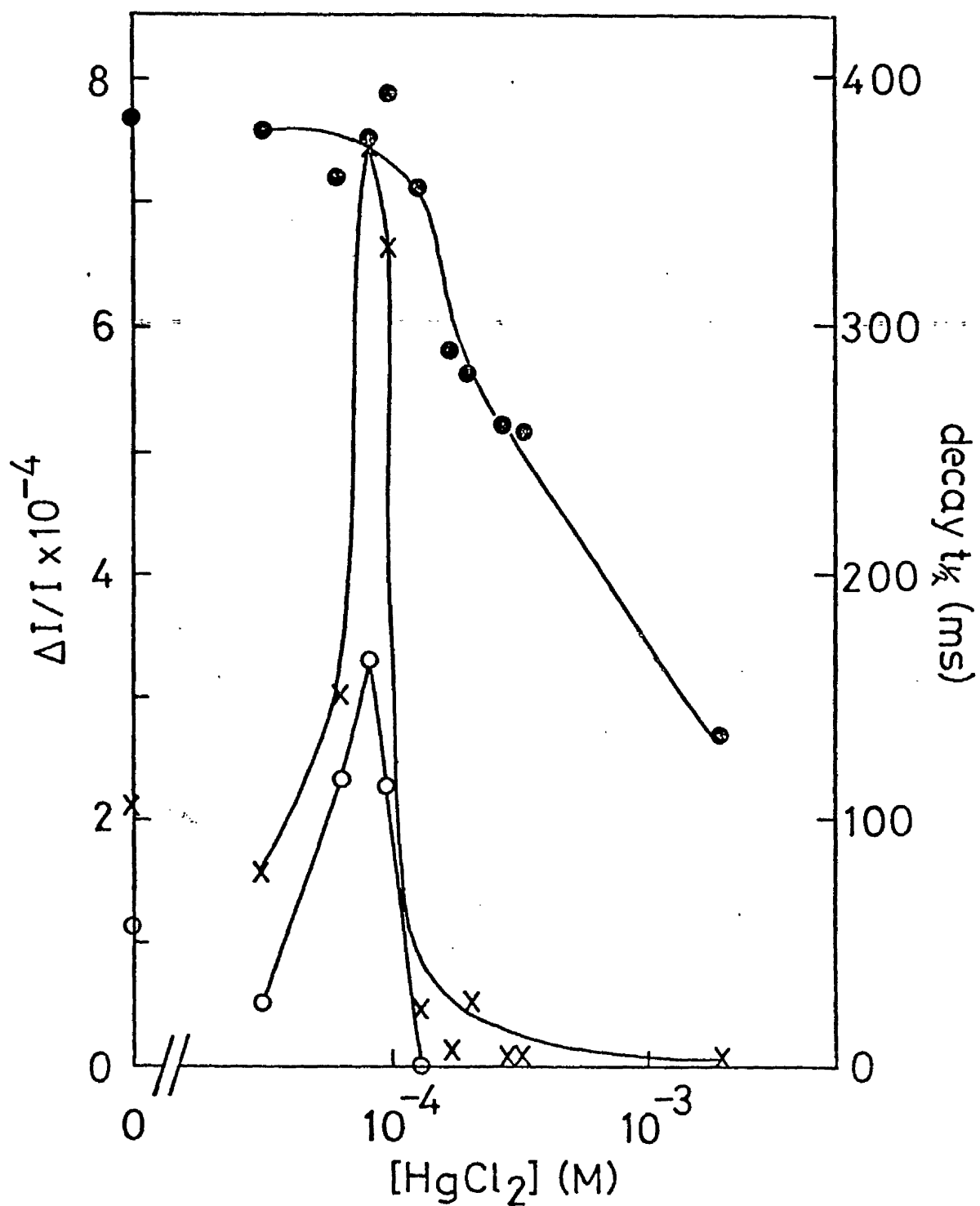


Figure 34. The effect of increasing concentrations of HgCl_2 on the fast phase (●), slow phase (o) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella* after 10 minutes dark incubation. Each point represents the accumulated average of 32 sweeps at a flash frequency of 0.5 Hz.

The experiments with KCN and HgCl_2 suggested that both compounds might be acting as uncouplers of sorts. In order to find out whether non-phenolic uncouplers were also capable of eliciting similar behaviour to those compounds tested so far it was decided to investigate the effect of NH_4Cl on the 515 absorption change and oxygen evolution. According to McCarty (1969) ammonia penetrates the thylakoid membrane in response to the light-induced proton uptake (section 1.4) and is then protonated in the acid intra-thylakoid space. Since the thylakoid membrane is envisaged as being impermeable to NH_4^+ ions, the uptake of NH_3 would lead to a selective dissipation of the proton gradient without effecting the membrane potential across the thylakoid membrane. The other phenolic uncouplers are envisaged to dissipate both components of the high energy state (see section 1.4). However, no effect of NH_4Cl , even at concentrations as high as $3.3 \times 10^{-2}\text{M}$ was detected on the oxygen evolution and 515 absorption change in Chlorella indicating that the compound was not penetrating the algal chloroplast and thus no conclusion could be drawn.

3.1.9 The Effect of CCCP and KCN on the Kinetics of Cytochrome f and Cytochrome b₅₆₃

A number of authors have recently suggested that the extra electrogenic step responsible for the slow phase of absorption increase at 515nm is intimately linked to the passage of electrons through the inter-system pool of electron carriers in the thylakoid membrane (see section 1.5.3.5.1) either via a cyclic flow of electrons or, as now seems more likely via some type of modified Q cycle. If this is the case then it should be possible to monitor changes in electron transfer rates through this section of the electron transport chain by studying the oxidation-reduction kinetics of cytochrome f and cytochrome b₅₆₃. Both of these cytochromes have been incorporated in various possible mechanisms to explain the extra electrogenic step and a number of authors have correlated their redox kinetics with the rise of the slow phase (Bourges-Bocquet, 1977b, 1980 and 1981; Velthuys, 1980a; Slovacek et al. 1979) (see section 1.5.3.5.1 for further detail).

Thus it was decided to study the kinetics of cytochrome f and cytochrome b₅₆₃ under control conditions and under conditions of maximal slow phase, stimulated by the addition of 0.8 μ M CCCP and as a comparison 10^{-4} M KCN. In addition, the effect of uncoupling concentrations of CCCP (10^{-4} M) was also included for comparison with the low uncoupler effect.

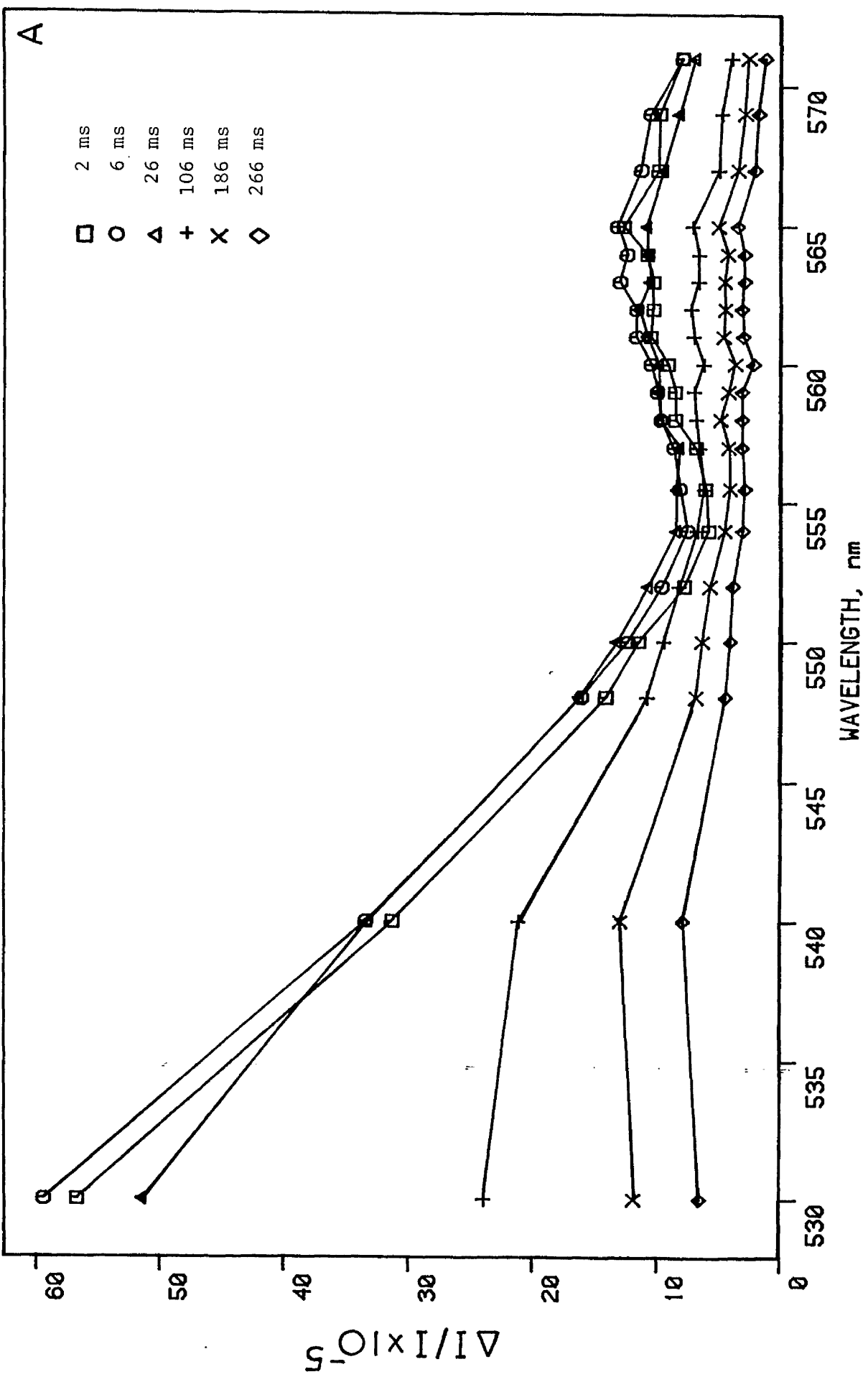
The redox kinetics of cytochrome f and cytochrome b₅₆₃ may be monitored by the absorption changes of their respective α bands at 554nm and 564nm. However, at these wavelengths there is still some absorption change contribution due to the electrochromic effect as was pointed out earlier in the results concerning the light minus dark difference spectra of the 515 absorption change (Figure 24). Thus before the true redox kinetics of the two cytochromes may be observed it is necessary to subtract a proportion of the signal assumed to be due to the electrochromic effect (Dolan and Hind, 1974; Slovacek et al. 1979; Velthuys 1979; Bourges-Bocquet 1977a). This digital subtraction of one signal from another relatively small one has proved difficult. A number of the different reported techniques were tried, but found to have various inherent problems. The major problem was not in accumulating the signals at the different wavelengths, but in adjudging the correct amount of the electrochromic signal which was to be subtracted from the signals observed at the wavelengths of the cytochrome bands. Small variations in the proportion of the electrochromic signal removed yielded very different signals for the cytochrome time courses and an examination of the apparent cytochrome absorption changes in the literature obtained by various subtraction techniques would seem to concur with this.

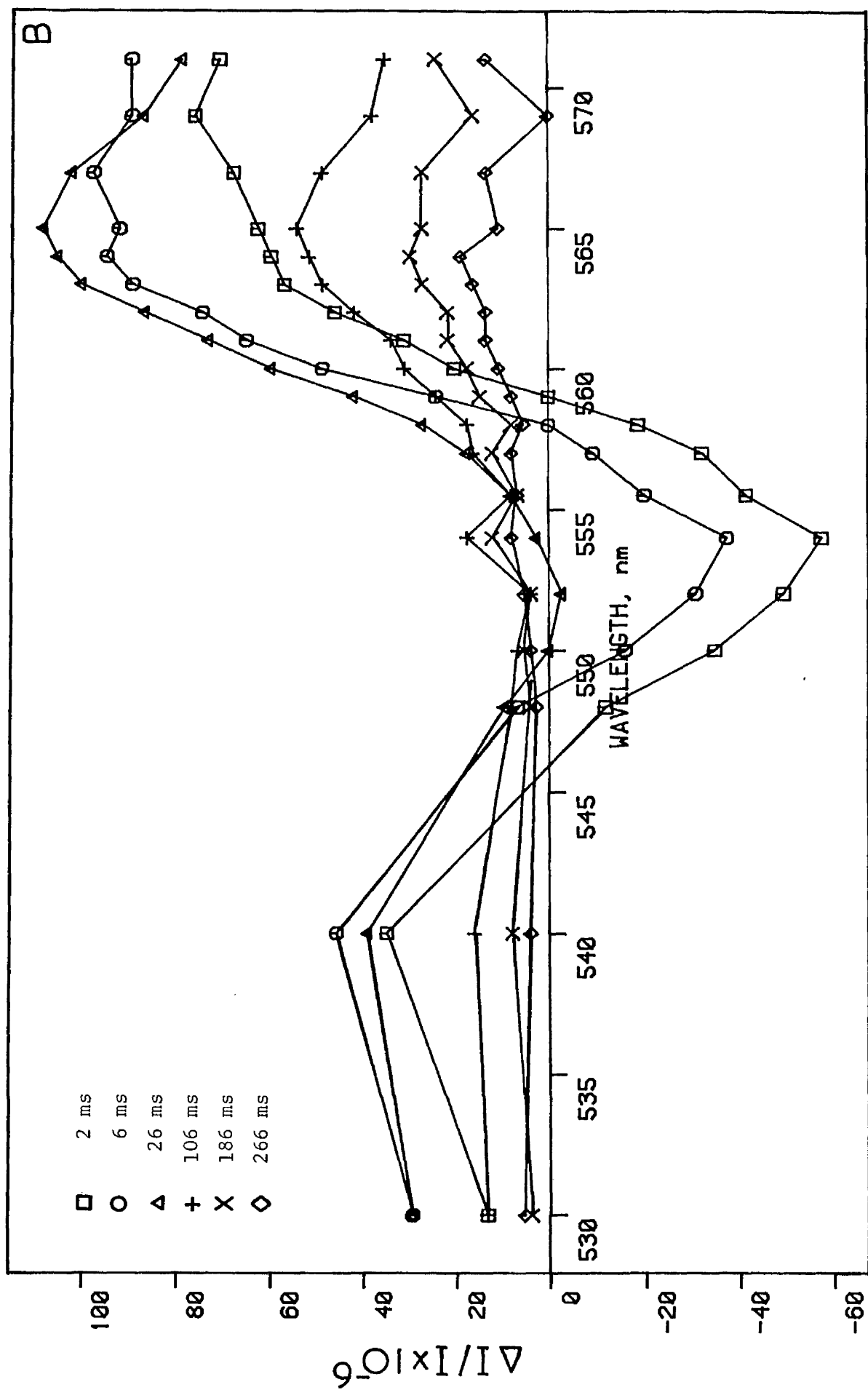
After spending a great deal of time investigating the various techniques a method was employed which was similar to that originally used by Dolan and Hind (1974) although modified to incorporate various ideas of Bourges - Bocquet (1977a). However it should be stressed that all the methods investigated had shortcomings and the one employed here seemed to be the most correct, but the cytochrome time courses obtained should be treated with discretion.

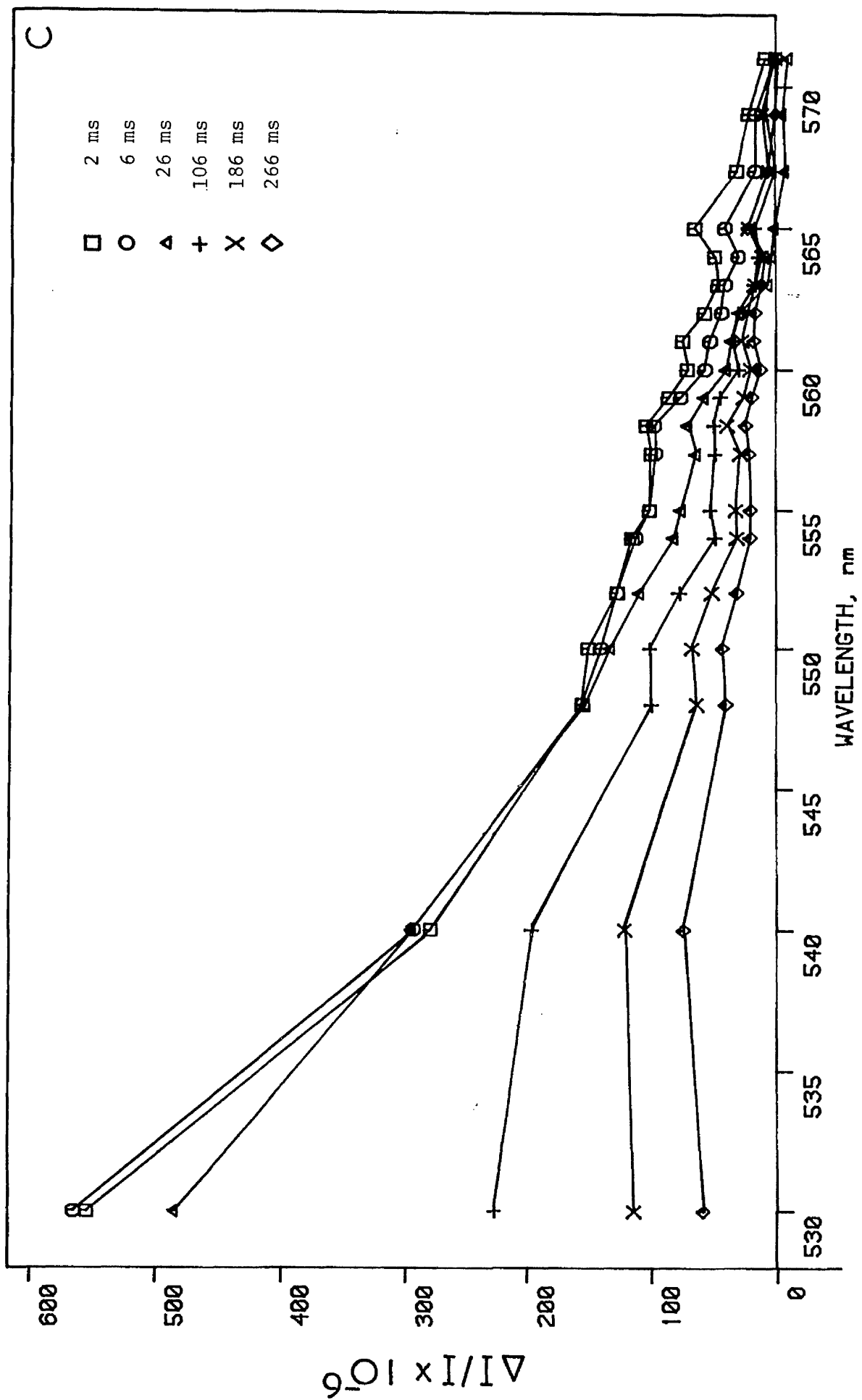
3.1.9.1 The Derivation of the Cytochrome Absorption Changes

Figure 35A shows a time-resolved light minus dark difference spectrum for Chlorella in the cytochrome absorption region from 530nm to 570nm on the wing of the large positive absorption peak at about 515nm which is attributed to the electrochromic effect (see Figure 24 and section 1.5.3). The spectra were obtained at six different times after the flash, between about 2 and 266 msec. It can be seen that the cytochrome changes are superimposed upon the tail of the electrochromic absorption change as a slight trough at 554nm and a slight peak at 564nm. This is particularly noticeable in the spectra obtained a short time after the flash (2, 6 and 26 msec after the flash) but not in the later spectra (106, 186 and 266 msec) suggesting that the cytochrome absorption changes are over before 106 msec, in agreement with Bourges-Bocquet (1977a). Figure 35B shows a similar time-resolved light minus dark difference spectrum for Chlorella but with the electrochromic absorption component removed by the presence of a strongly uncoupling concentration ($10^{-3}M$) of CCCP. In this spectra the negative peak of cytochrome f at 554nm becomes much more apparent as does the positive peak at 564nm due to cytochrome b₅₆₃. Under these uncoupled conditions the cytochrome changes are observed to have relaxed by 106 msec. By digitally subtracting the uncoupled spectra (Figure 35B) from the control spectra (Figure 35A) corrected spectra of the electrochromic absorption change are obtained (Figure 35C) with no superimposed absorption changes from the cytochromes at 554 and 564nm.

Figure 35. Light minus dark difference spectra of the flash-induced absorption change from 530 nm - 571 nm in Chlorella after 5 minutes dark adaption at different times after the actinic flash. A, no additions, showing the uncorrected spectra; B, with the addition of 10^{-3} M CCCP to remove the electro-chromic component; C, B subtracted from A to give the corrected spectra of the electrochromic component. Each point represents the accumulated average of 512 sweeps at a flash frequency of 0.5 Hz.







From these corrected spectra the contribution of the electrochromic absorption change at each wavelength and at different times after the flash can be observed. Therefore, assuming the cytochrome changes have relaxed by 100 msec (Bourges-Bocquet, 1977a; Slovacek et al. 1979) as evident from Figure 35A and 35B, and knowing the size of the electrochromic effect at each wavelength at 100 msec (Figure 35C) and the kinetics of the electrochromic shift (e.g. the absorption change at 530nm) the fraction of absorption change at 530nm to be subtracted from any other wavelength is obtained from the extent of the two absorption changes 100 msec after the flash taken from Figure 35C. Therefore, the fraction of 530nm subtracted from 554nm and 564nm to yield the true cytochrome redox absorption changes is $(\Delta A_{554} / \Delta A_{530})_{100 \text{ msec}}$ and $(\Delta A_{564} / \Delta A_{530})_{100 \text{ msec}}$ for cytochrome f and cytochrome b₅₆₃ respectively. From Figure 35C these respective fractions are 0.212 and 0.059, which are the fractions of the electrochromic change at 530nm removed from the absorption changes at 554 and 564 in order to observe the kinetics seen in Figures 36 and 37. When employing this technique it is assumed that the kinetics of the electrochromic absorption change as seen at 530nm do not change at different wavelengths. This assumption as seen from Figure 35C is moderately well-founded, but as the wavelength increased so the size of the electrochromic absorption component became progressively small and hence the signal-to-noise ratio decreased. Thus the kinetics of the longer wavelength time courses are probably less accurate, as are the time-resolved spectra.

3.1.9.2 The Effect of CCCP on the Flash-Induced Absorption Changes Attributed to Cytochrome f and Cytochrome b₅₆₃

Assuming that the absorption changes derived at 554nm and 564nm indicate the redox changes of the cytochromes, the effects of varying the concentration of CCCP as in Figure 20A were investigated on Chlorella dark adapted for 5 minutes.

For reasons of better presentation only the time courses under (i) control conditions, (ii) conditions of maximum stimulation of the slow phase by $0.8\mu\text{M}$ CCCP and (iii) uncoupled conditions by 10^{-4}M CCCP, are depicted. Figure 36 shows the time courses at 530nm, and 554nm and 564nm after correction for the electrochromic effect under the 3 conditions. The cytochrome f traces show a fast oxidation within a time shorter than the time resolution of the method but with a reduction time that is dependent on the CCCP concentration. Under control conditions the cytochrome f signal shows a return to the reduced state after approximately 100 msec, but under conditions of maximal slow phase (+ $0.8\mu\text{M}$ CCCP) the reduction rate is slowed quite noticeably. When strongly uncoupled conditions exist (+ 10^{-4}M CCCP) the rate of reduction is markedly increased suggesting that by removing the energetic burden of phosphorylation from the electron transfer chain, uncoupling concentrations of CCCP induce increased rates of electron transport through cytochrome f. The slowing down of the cytochrome f reduction by low uncoupler concentrations is unexpected and differs from other reports which suggest that the slow phase is associated with increased rates of electron transfer through cytochrome f. (Slovacek et al. 1979; Bourges-Bocquet, 1977b; Velthuys, 1978).

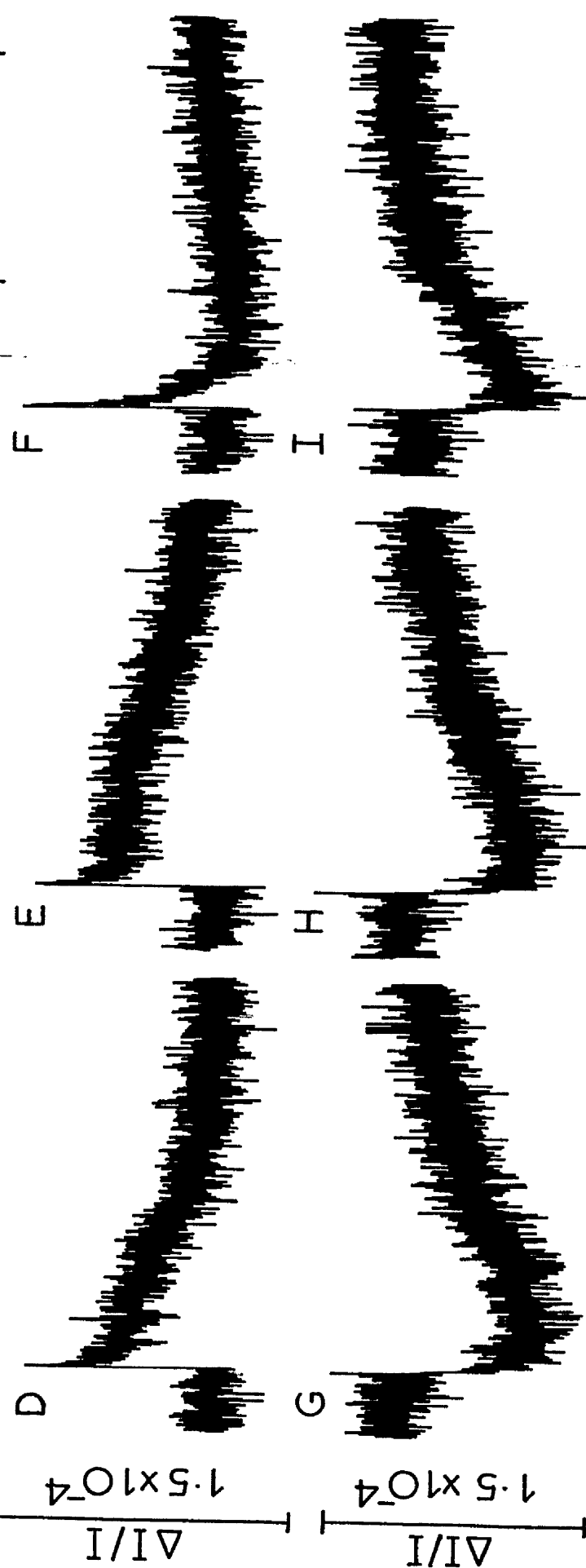
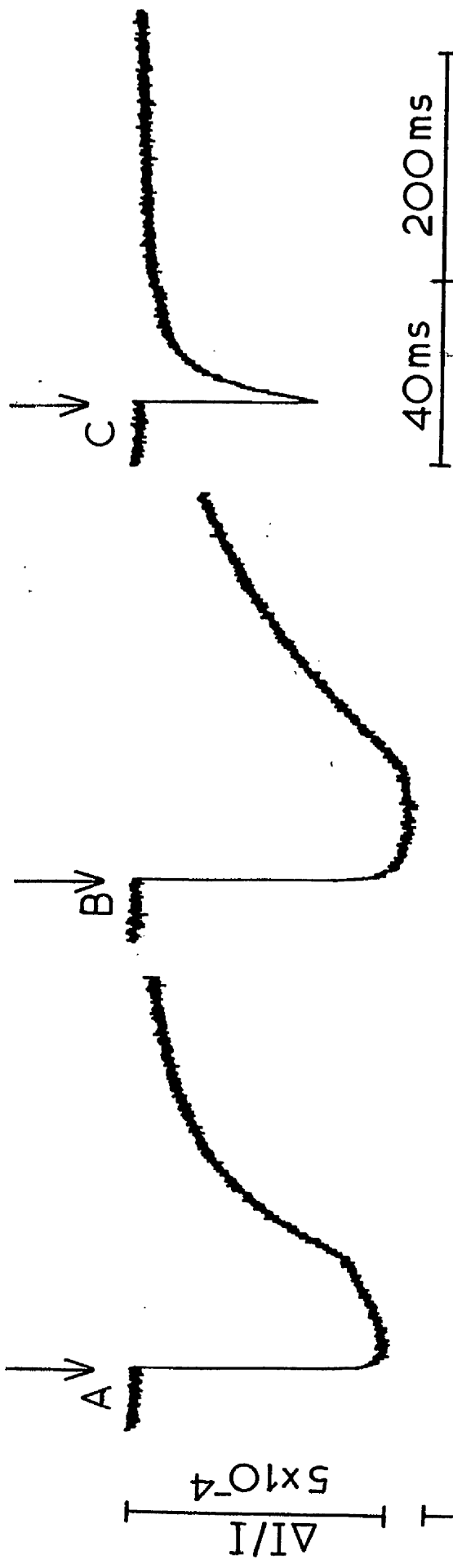
The cytochrome b₅₆₃ changes show a reduction time of approximately 10msec and oxidation time which again varies according to the uncoupler concentration. When the slow phase is maximised very little difference in the reoxidation kinetics is seen but under uncoupling conditions the rate is increased markedly. Slovacek et al.(1979) have correlated an increased rate of reoxidation of cytochrome b₅₆₃ with the slow phase in chloroplasts but Bourges-Bocquet (1980) has not been able to resolve redox changes of the cytochrome in Chlorella.

Thus these results show that unlike most of the literature the stimulation of maximal slow phase is correlated with slower or unchanged cytochrome turnovers.

Figure 36. Kinetic traces of the flash-induced absorption changes in Chlorella attributed to: A, B, C, the electrochromic effect as measured at 530 nm; D, E, F, the oxidation-reduction kinetics of cytochrome f measured as $(\Delta I_{554}/I_{554}) - 2.12 \times (\Delta I_{530}/I_{530})$; G, H, I, the oxidation-reduction kinetics of cytochrome b₅₆₃ measured as $(\Delta I_{564}/I_{564}) - 0.059 \times (\Delta I_{530}/I_{530})$. A, D, G, no additions; B, E, H, with the addition of $0.8 \mu\text{M}$ CCCP; C, F, I, with the addition of 10^{-4} M CCCP. Each trace represents the accumulated average of 512 sweeps at a flash frequency of 0.5 Hz.

The transient recorder was used in the split time base mode
(100msec/400msec)

The flash was fired at the point indicated by the arrow.



3.1.9.3 The Effect of KCN on the Cytochrome f and Cytochrome b₅₆₃ Absorption Changes

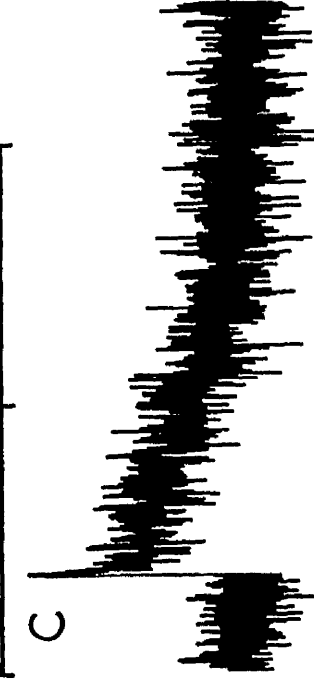
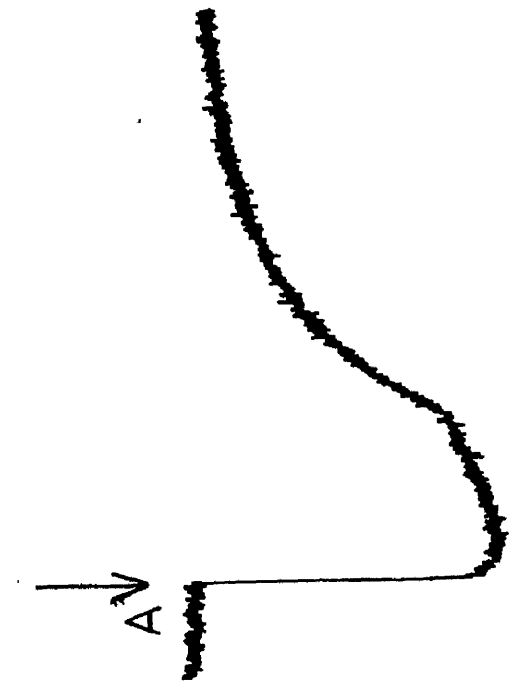
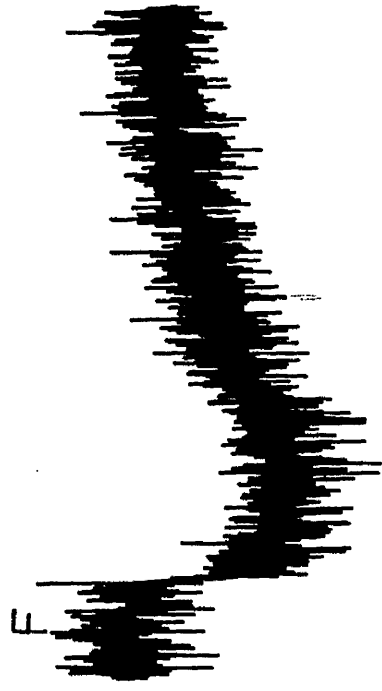
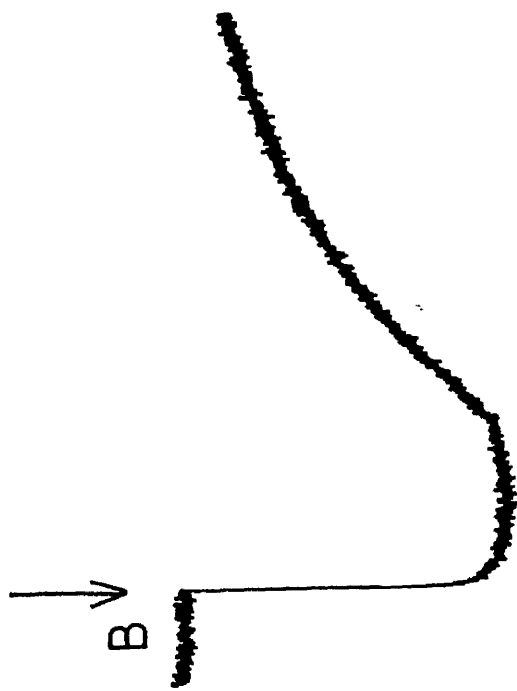
As with the CCCP results, the data obtained for the effect of KCN on the cytochrome absorption changes must be treated with some caution. In order to verify the slowing of the cytochrome f reduction and the lack of effect on cytochrome b₅₆₃ caused by stimulation of the slow phase by 0.8 μ M CCCP, the effect of stimulating the slow phase with varying concentration of a different compound, KCN (see Figure 32) was investigated. KCN was not found to uncouple at high concentrations so unlike the CCCP data no high concentration data are presented. As with the CCCP results only the cytochrome traces of (i) control and (ii) maximal stimulation of the slow phase (10⁻⁴M KCN) are depicted for reasons of presentation, not the changes of the complete concentration range. Figure 37 shows the effect of 10⁻⁴M KCN on the electrochromic effect as observed at 530nm, and the absorption changes at 554nm and 564nm which are attributed to the redox changes of cytochrome f and b₅₆₃ respectively (after correction for electrochromic contribution). The data show similar trends to the CCCP data; the stimulation of the slow phase by addition of 10⁻⁴M KCN results in a decrease in the rate of rereduction of cytochrome f and very little difference in the cytochrome b₅₆₃ kinetics. Although no reports in the literature of the effect of KCN concentration on cytochrome changes in isolated chloroplasts or Chlorella could be found, the fact that stimulation of the slow phase results in a decreasing rate of cytochrome f rereduction is inconsistent with the literature, as with the CCCP data.

3.1.10 Deconvolution of the 515 Absorption Change Curves

In order quantitatively to analyse the stimulation of the slow phase and decrease in the rate of decay in Chlorella by low uncoupler concentrations and KCN and HgCl₂ it was decided to try to resolve the kinetic components by fitting a number of exponential curves to some of the experimental traces obtained (the data is shown in Table 2).

Figure 37. Kinetic traces of the flash-induced absorption changes in Chlorella attributed to; A, B, the electrochromic effect as measured at 530 nm; C, D, the oxidation-reduction kinetics of cytochrome f measured as $(\Delta I_{554}/I_{554}) - 2.12 \times (\Delta I_{530}/I_{530})$; E, F, the oxidation-reduction kinetics of cytochrome b₅₆₃ measured as $(\Delta I_{564}/I_{564}) - 0.059 \times (\Delta I_{530}/I_{530})$. A, C, E, no additions; B, D, F, with the addition of 10^{-4} M KCN. Each trace represents the accumulated average of 512 sweeps at a flash frequency of 0.5 Hz. The transient recorder was used in the split time base mode (100msec/400msec)

The flash was fired at the point indicated by the arrow.



$\Delta I/I$
 5×10^{-4}

$\Delta I/I$
 1.5×10^{-4}

$\Delta I/I$
 1.5×10^{-4}

40ms 150ms

Table 2

Deconvolution of Typical Experimental Traces of the Flash-Induced Electrochromic Absorption Change under Control Conditions and in the Presence of 0.8 μ M CCCP, 10⁻⁴M KCN and 10⁻⁴M HgCl₂ according to a Linear Combination of Two Exponentials: $\Delta A(t) = \{ A + B(1 - \exp^{-k t}) \} \exp^{-t/\tau}$

Treatment	A ($\Delta I/I \times 10^{-4}$)	B ($\Delta I/I \times 10^{-4}$)	k (msec)	τ (msec)
(a) Control	4.29 \pm 0.098	1.15 \pm 0.094	2.83 \pm 0.49	57.20 \pm 0.84
(b) + 0.8 μ M CCCP	4.60 \pm 0.05	1.37 \pm 0.05	5.32 \pm 0.47	130.00 \pm 1.69
(b)/(a) x 100%	107.1	119.0	188.0	227.3
(c) Control	4.25 \pm 0.13	1.07 \pm 0.12	2.44 \pm 0.56	54.06 \pm 0.93
(d) + 10 ⁻⁴ M KCN	4.38 \pm 0.05	1.41 \pm 0.05	5.10 \pm 0.46	97.34 \pm 1.15
(d)/(c) x 100%	103.0	132.5	208.9	180.0
(e) Control	7.84 \pm 0.52	2.32 \pm 0.51	6.14 \pm 3.65	185.71 \pm 7.41
(f) + 10 ⁻⁴ M HgCl ₂	7.71 \pm 0.47	3.42 \pm 0.46	6.93 \pm 2.03	356.43 \pm 12.45
(f)/(e) x 100%	98.3	147.0	112.9	193.0

Table 3

Deconvolution of Typical Experimental Traces of the Flash-Induced Electrochromic Absorption Change in the Presence of 10⁻⁴M CCCP, according to a Linear Combination of Two Exponentials: $\Delta A(t) = A \exp^{-k t} + B \exp^{-t/\tau}$

Treatment	A ($\Delta I/I \times 10^{-4}$)	B ($\Delta I/I \times 10^{-5}$)	k (msec)	τ (msec)
+ 10 ⁻⁴ M CCCP	3.35 \pm 0.043	4.0 \pm 0.028	3.56 \pm 0.1	59.97 \pm 6.9

* Footnote to Table 2. It should be noted that the half-rise time of the slow phase is increased both by 0.8 μ M CCCP and by 10⁻⁴M KCN.

The specific traces analysed in Table 2 are those obtained at 530nm for the experiments investigating the effects of CCCP and KCN concentration on the cytochrome redox changes shown in Figures 36 and 37 and also some typical mercuric chloride data obtained from the experiments shown in Figure 34. In order to fit the curves optimally various types of exponential deconvolution by a least squares fit computer analysis were attempted. The type which fitted the control and stimulated traces best was $\Delta A(t) = \{ A + B (1 - \exp -k_1 t) \} \exp -k_2 t$ where A and B are the extents of the fast and slow phase and k_1 and k_2 are rate constants for the rate of increase of the slow phase and the rate of decay for the overall absorption change respectively. The values obtained from the deconvolutions are displayed in Table 2 as changes in absorption for the extents and half times (msec) for the decay rates. The errors are depicted as standard deviations not standard errors. The data indicate that the addition of concentrations of $0.8 \mu\text{M}$ CCCP, 10^{-4}M KCN and 10^{-4}M HgCl_2 do not greatly effect the fast phase of increase and slight increases of the extents of the slow phases occur whereas a marked decrease in the decay rates do occur. This suggests that although increases in the slow phases do occur the stimulations seen may only be a consequence of the slow phase being more easily seen when the rate of decay is slowed down.

The effects of uncoupling concentrations of CCCP (10^{-4}M) were also investigated in this manner but would not fit to the above exponential equation and were found to fit better to the equation $\Delta A(t) = A \exp -k_1 t + B \exp -k_2 t$. The values obtained are shown in Table 3 and indicate a markedly increased rate of decay, a smaller fast phase and no slow phase. This may be explained by the fact that the presence of strongly uncoupling concentrations would lead to such an increased counter flux of ions (protons) back through the membrane that a part of the field set up by charge separation at the reaction centres would have decayed within a time shorter than the resolution of measuring technique.

To illustrate the fitting accuracy of the various deconvolution techniques the curves which were fitted to the CCCP data are displayed in Figure 38. The traces show a reasonably close fit to each of the curves, although it is stressed that none of the curves are identical fits but were the best which could be obtained.

3.1.11 Studies with Isolated Chloroplast Preparations

A major problem in the experiments carried out so far had been the impermeability of the algal cells, preventing many compounds such as inhibitors, redox dyes, uncouplers, electron acceptors and donors from entering the chloroplast and its thylakoid membranes. In order to gain further information on the electrogenic mechanisms responsible for the slow phase, it was deemed necessary to try and isolate in vitro photosynthetic systems. It was hoped that these preparations would exhibit as full a photosynthetic activity as the in vivo algal cells, particularly in possessing the slower phase of absorption increase at 515nm, which a number of earlier chloroplast preparations had not, but without the problem of a permeability barrier isolating the various substrates and inhibitors from the thylakoid surface.

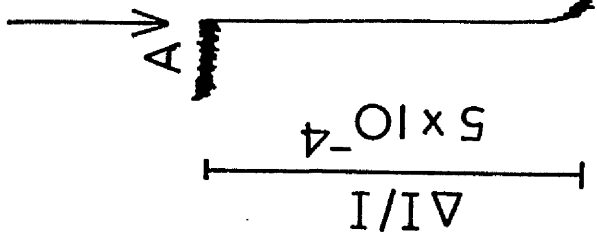
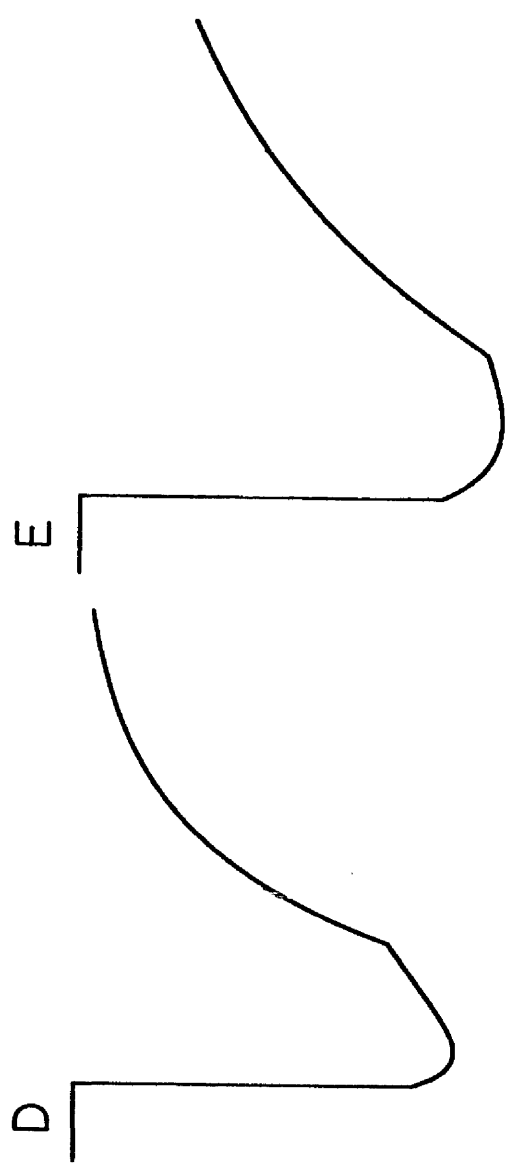
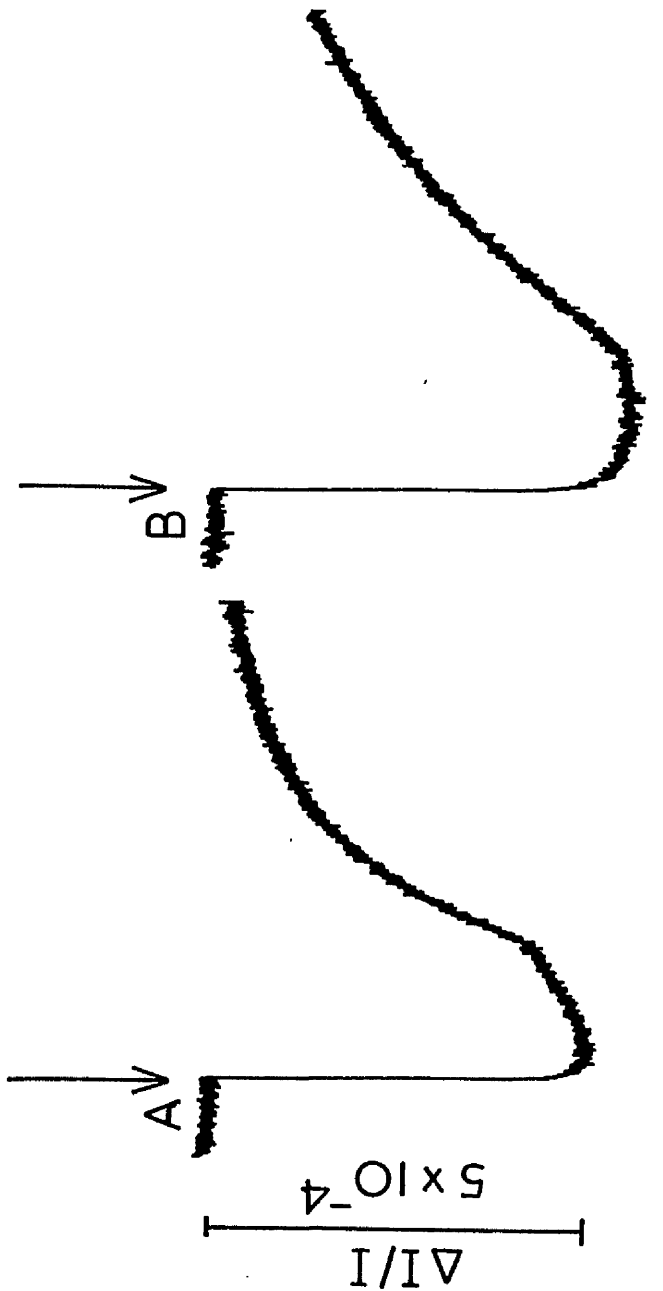
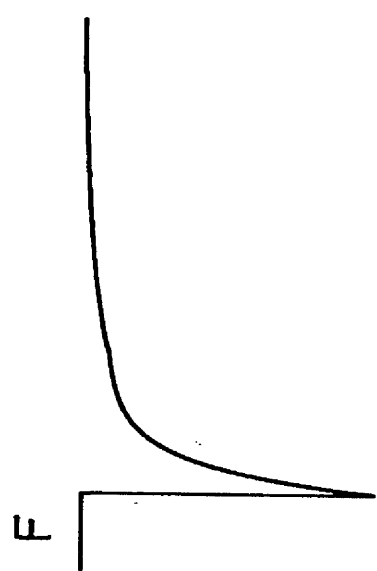
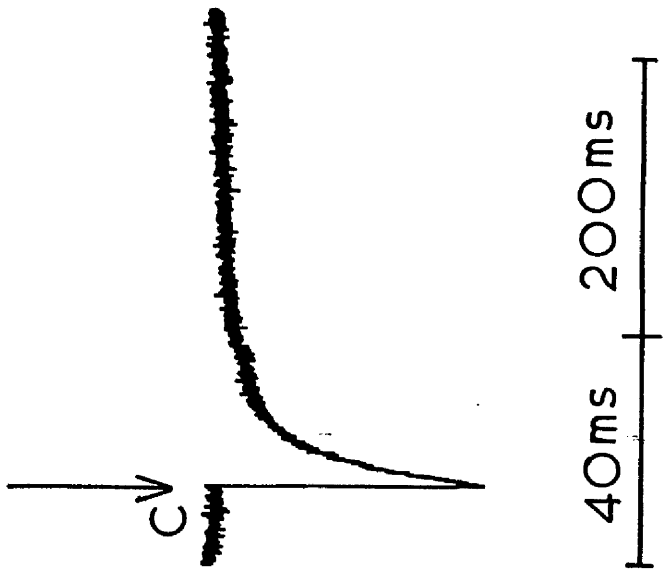
3.1.11.1 Isolation of Intact Chloroplasts

In order for the slow phase of absorption increase seen in intact algae to be present in chloroplasts it was thought necessary to have a fairly high level of envelope intactness (Horvath et al. 1979) i.e. the slow phase was dependent on the preservation of the stromal environment around the thylakoids. With this objective, chloroplast preparations were carefully obtained, initially from hydroponically grown spinach.

Figure 38. Illustration of the curves fitted by a least squares fit programme to the experimental traces of the effect of CCCP on the electrochromic absorption change obtained in figure 36. Traces A and B fitted to the equation $\Delta A(t) = \{A + B(1 - \exp(-kt))\} \exp(-lt)$ to give curves D and E; trace C fitted to the equation $\Delta A(t) = A \exp(-kt) + B \exp(-lt)$ to give curve F. A, no additions; B, with the addition of $0.8 \mu\text{M}$ CCCP; C, with the addition of 10^{-4}M CCCP. Each experimental trace represents the accumulated average of 512 flashes at a flash frequency of 0.5 Hz.

The experimental traces were obtained using the transient recorder in the split time base mode (100msec/400msec).

The flash was fired at the point indicated by the arrow.



However, no spinach chloroplast preparation could be obtained which showed slow phase and so a similar isolation was attempted from pea plants. Using peas it became possible to obtain pea chloroplasts routinely with an intactness between 55 and 75% according to the ferricyanide assay and which did show slow phase. However, it should be noted that the intactness was variable and therefore absolute values of slow phase and decay rate varied from one preparation to another.

3.1.11.2 The Effect of Pre-Illumination on Intact Chloroplasts

It has been reported by Velthuys (1978) that for optimal slow phase to be present in chloroplasts some form of pre-illumination is necessary. Thus to obtain maximal slow phase for the ensuing experiments the effect of varying pre-illumination times on isolated chloroplasts was investigated. Figure 39(A) shows that maximal slow phase was obtained after approximately 10 seconds of white pre-illumination (intensity circa 20 Wm^{-2}). Greater or lesser amounts of pre-illumination decreased the extent of the slow phase. The fast phase was relatively unaffected by the light pre-treatment.

Velthuys (1978) suggested that the reason for pre-illumination optimising the slow phase extent was that extensive reduction of the plastoquinone pool is a pre-requisite condition for the slow phase to be generated. From the data of Figure 39(A) it is suggested that the reduction of the pool is optimised after 10 seconds illumination under these experimental conditions for intact chloroplasts. Bourges-Bocquet (1980) has reported that another unknown component has to be reduced not plastoquinone, for the slow phase to be present in Chlorella.

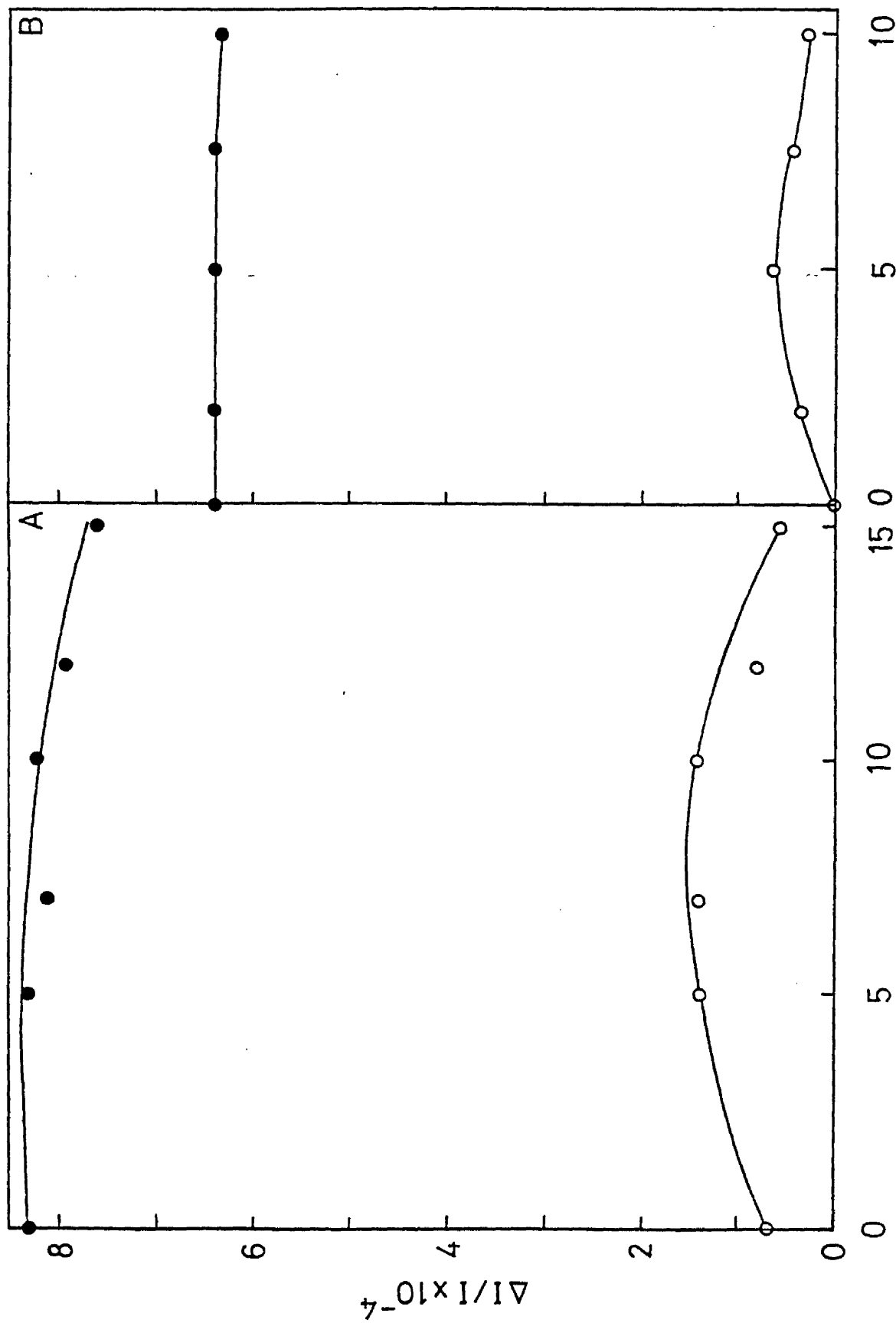


Figure 39. The effect of pre-illumination (white light, giving an intensity of 20 Wm^{-2}) on the fast phase (●) and slow phase of the flash-induced absorption change at 515 nm. A, in intact pea chloroplasts; B, in pea chloroplasts osmotically shocked in 10 mM MgCl_2 , 1 mM TRIS, pH 8.2. Each point represents the accumulated average of 32 sweeps at a flash frequency of 0.5 Hz.

3.1.11.3 The Effect of CCCP Concentration on Intact Chloroplasts

It was hoped that the stimulation of the slow phase obtained with low uncoupler concentrations in Chlorella would be repeated in intact chloroplasts so that this effect might be investigated more thoroughly without the problems of impermeability towards the various inhibitors etc which hampered the studies in Chlorella. Figures 40 and 41 show the effect of CCCP concentration over the range of 10^{-11} - 10^{-5} M on both pre-illuminated (10 seconds, white light giving an intensity of 20 Wm^{-2}) and non pre-illuminated intact chloroplasts respectively. Both sets of data show that as in the case of the bacterial chromatophores (Figure 19B) there was neither a stimulation of the slow phase of absorption increase nor a decrease in the rate of decay as had been hoped. Increasing concentrations of CCCP merely induced a gradual decrease in extent of both the slow phase and fast phase and in addition, accelerated the rate of decay in both experiments. Thus in contrast to the effects seen with Chlorella, CCCP acts as a classic uncoupler only, in intact chloroplasts. The data is in contrast to the results of Slovacek et al. (1979) who reported that a number of uncouplers, i.e. $0.5 \mu\text{M}$ CCCP, $1.0 \mu\text{M}$ nigericin, $3.3 \mu\text{M}$ NH_4Cl and $2 \mu\text{M}$ monensin, induced slow phase in intact spinach chloroplasts. They interpreted this data by suggesting the slow phase is therefore associated with increased electron transfer through the inter-system chain associated with cyclic electron flow, as in addition to stimulating the slow phase, the uncouplers also increased the rates of turnover of cytochrome f and cytochrome b₅₆₃. This difference in results may suggest that our chloroplast preparations were under different conditions either in redox poisoning of various electron transport components, structural integrity of the chloroplast components, or by possible differences in the technical procedures of the experiments.

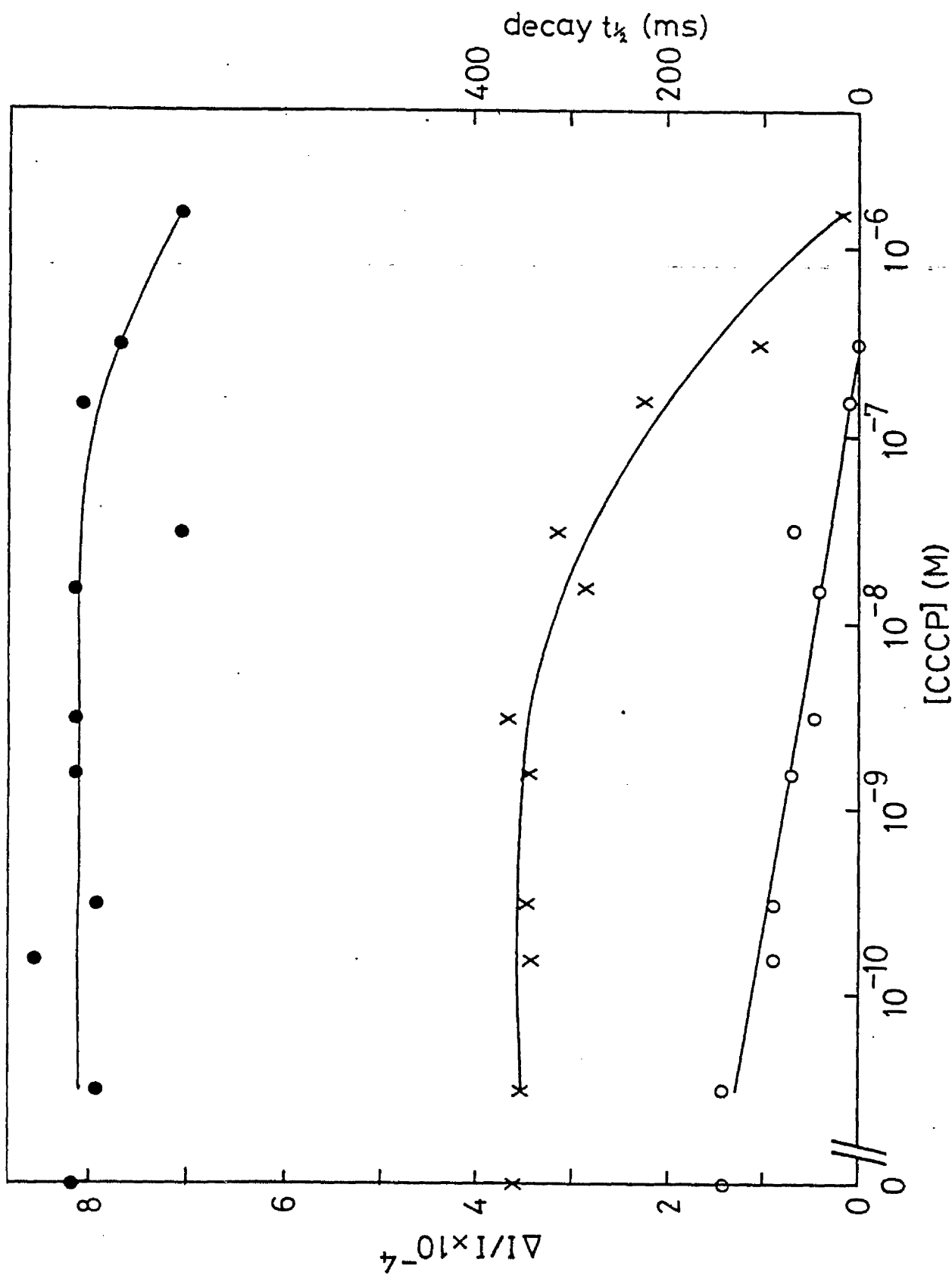


Figure 40. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in intact pea chloroplasts, pre-illuminated for 10 seconds (white light giving an intensity of 20 Wm^{-2}). Each point represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz.

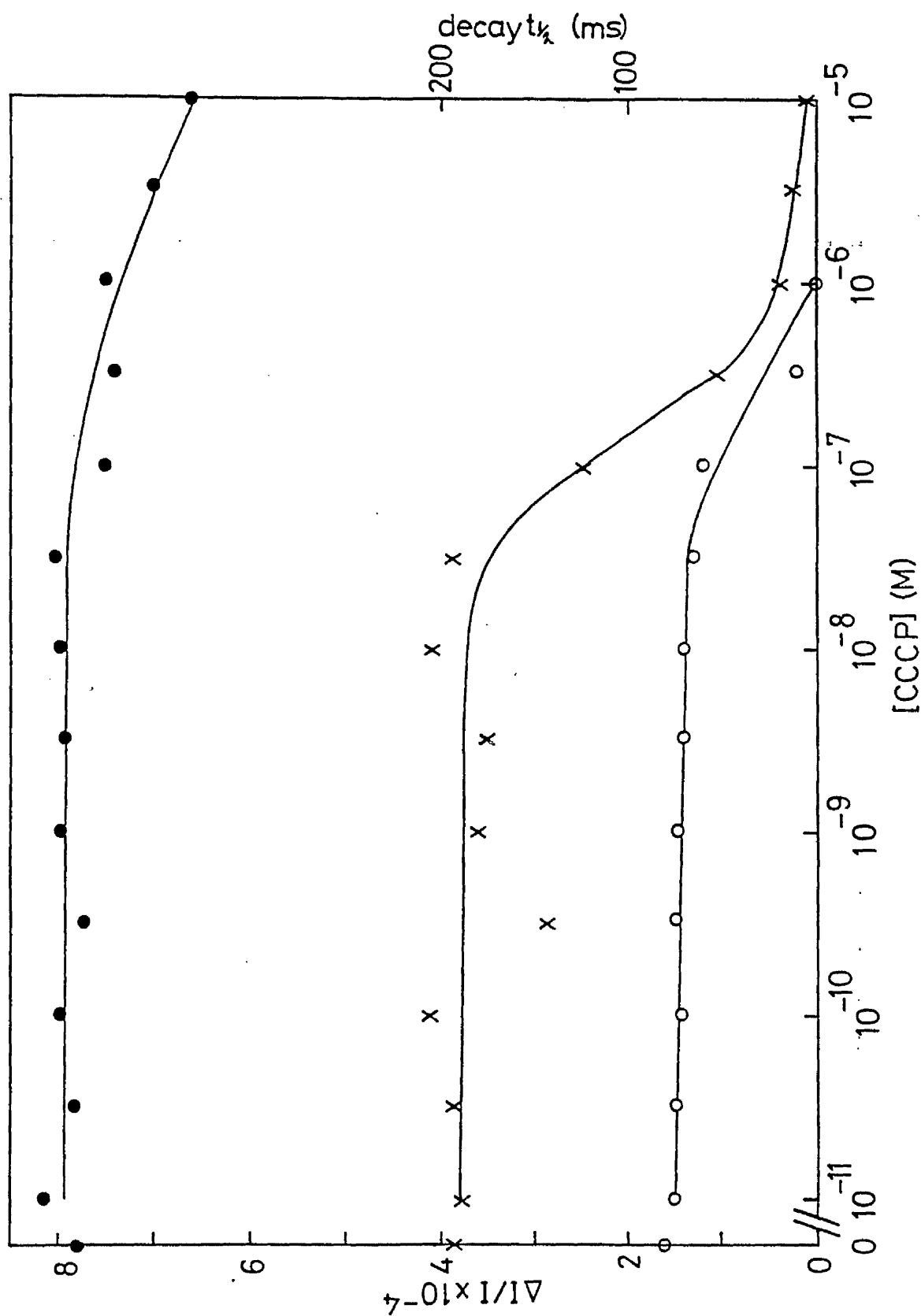


Figure 41. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in non pre-illuminated intact pea chloroplasts. Each point represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz.

3.1.11.4 Isolation of Shocked Chloroplasts Exhibiting Slow Phase

As the stimulatory effect of CCCP could not be obtained with intact chloroplast preparations, one further in vitro system was investigated in hope that this might provide a system for investigating the CCCP effect without the permeability problems of Chlorella, as described in the section on intact chloroplasts. Previously it had been assumed that osmotically shocked chloroplasts had lost the soluble stromal or loosely bound thylakoid components necessary for the retention of both a slow phase of absorption increase at 515nm and a normal physiological rate of decay (see Figure 19(A)). However, recent work by Shahak et al. (1980) had suggested that cyclic electron flow and the slow phase may be partially retained in osmotically shocked chloroplasts if Mg^{2+} was present during osmotic rupture of the chloroplast outer envelope. This procedure provided a means of restoring the slow phase without having to add back large quantities of exogenous electron carriers, such as ferredoxin, thought to be the components of the electron transport chain responsible for generating the slow electrogenic response, which are lost during osmotic shocking (Shahak et al. 1980). It was thought that data, showing the restoration of slow phase by addition of ferredoxin to shocked chloroplasts, (Shahak et al. 1980) pointed to a role of Mg^{2+} binding ferredoxin to the thylakoid particularly at the ferredoxin NADP reductase (see section 1.3.5) site as small amounts of Mg^{2+} increase the quantum yield of ferredoxin dependent NADP reduction (Marsho and Kok, 1974). Another possible mechanism for the Mg^{2+} -induced restoration of the slow phase might be by affecting the structure of the membrane by interacting with the membrane surface potential at the level of the fixed charges around the thylakoid. Divalent cations have been reported to influence a number of photosynthetic processes such as fluorescence and electron transport via their interaction with the diffuse charge layer surrounding the thylakoid (see Barber, 1976 for review).

Figure 42 shows typical traces of the 515 change obtained with chloroplasts: (A) intact, (B) osmotically shocked in H_2O and pre-illuminated for 5 sec, (C) osmotically shocked in 10mM $MgCl_2$ and dark-adapted for 5 minutes, (D) osmotically shocked in 10mM $MgCl_2$ and pre-illuminated for 5 sec. The traces show that chloroplasts which were osmotically shocked in the presence of 10mM $MgCl_2$ did show a slow phase of absorption increase at 515nm but only when the intact chloroplasts from which the shocked chloroplasts were derived were relatively intact (74% in this experiment) as assayed by the ferricyanide method (see section 2.2.1). In addition it was found that as in intact chloroplasts, pre-illumination was necessary for maximal slow phase to occur. This was further investigated in a similar manner to the intact chloroplasts in Figure 39(A) and the results shown in Figure 39(B). The data shows that less pre-illumination was necessary in shocked chloroplasts than in intact, about 5 seconds being optimal. Again as in intact chloroplasts too much pre-illumination decreased the extent of the slow phase. Like the intact chloroplasts this optimal pre-illumination indicated some form of optimum poising of the electron transport components was necessary for the slow phase to occur.

Although these studies agree with the proposal of Shahak et al. (1980) that Mg^{2+} is necessary for the slow phase, they also suggest that Mg^{2+} alone gives only a fraction of the slow phase obtained when the chloroplasts are pre-illuminated also. Shahak et al. (1980) pre-illuminated all their chloroplast preparations with 40 flashes before measuring absorption changes so it is unclear from their data whether Mg^{2+} alone is responsible for inducing slow phase in shocked chloroplasts. The data presented here would suggest both are necessary.

Electron micrographs of the various preparations for the above experiments are shown in Figure 43.

Figure 42. Typical examples of the flash-induced absorption change at 515 nm in intact and osmotically shocked pea-chloroplasts. A, intact (74%) chloroplasts after 10 seconds pre illumination (white light giving an intensity of 20 Wm^{-2}); B, chloroplasts osmotically shocked in H_2O followed by 5 seconds pre-illumination as in A; C, chloroplasts osmotically shocked in 10 mM MgCl_2 , 1 mM TRIS, pH 8.2, followed by 5 minutes dark-adaption; D, chloroplasts osmotically shocked in 10 mM MgCl_2 , 1 mM TRIS, pH 8.2, followed by 5 seconds pre-illumination as in A. Each trace represents the accumulated average of 32 sweeps at flash frequency of 0.5 Hz.

The transient recorder was used in the split time base mode (200msec/2sec)

The flash was fired at the point indicated by the arrow.

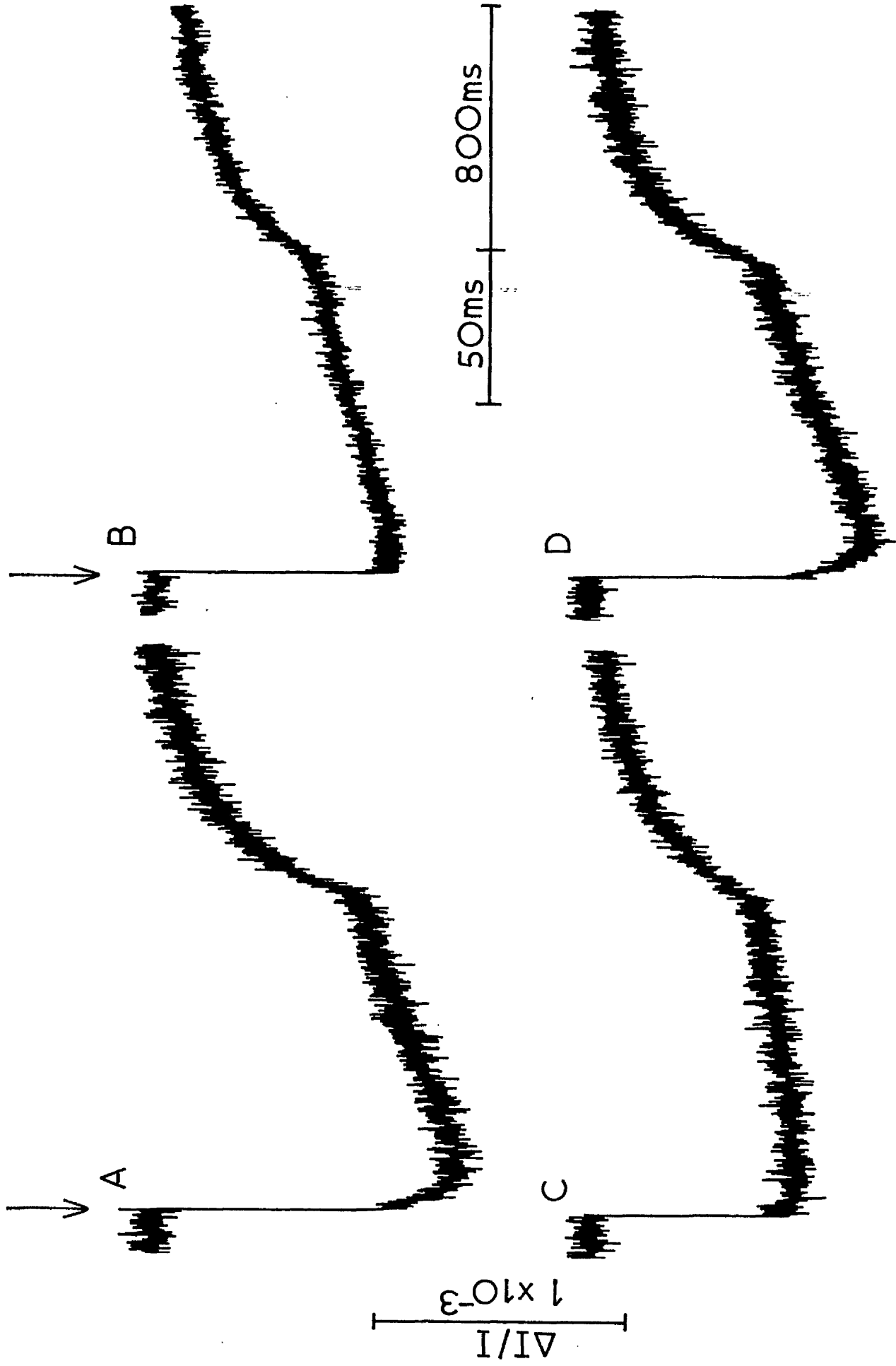
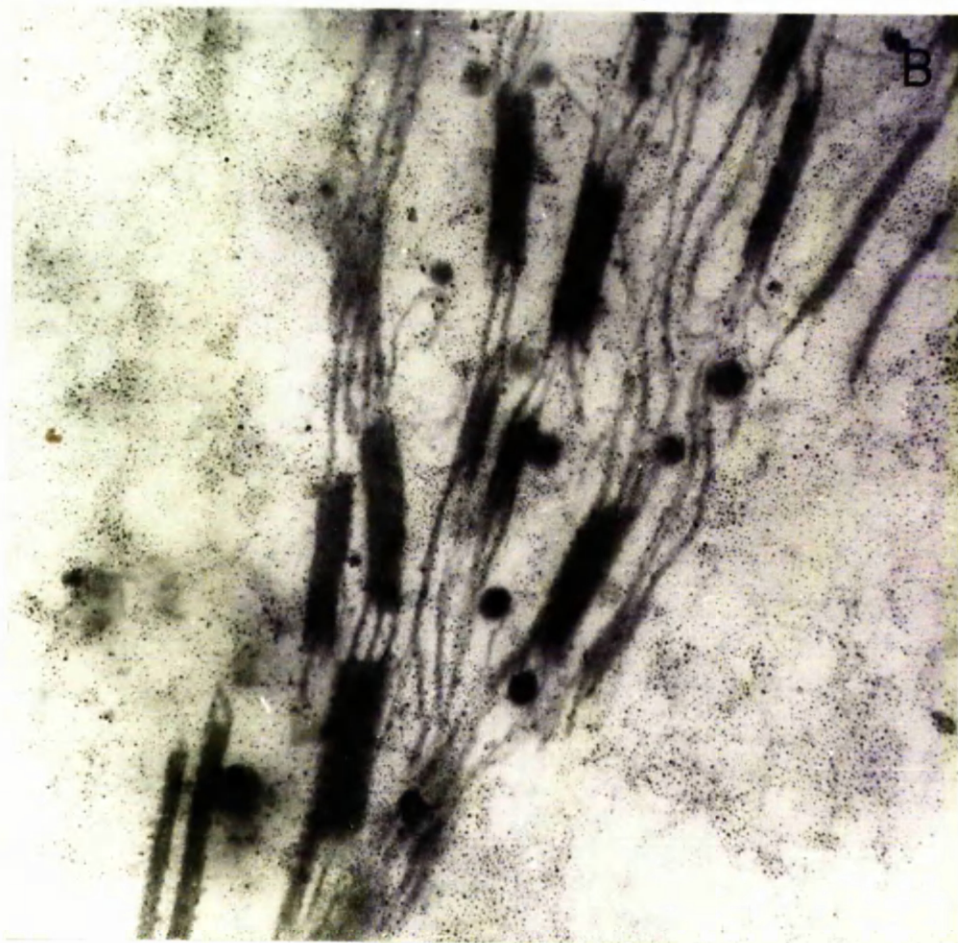
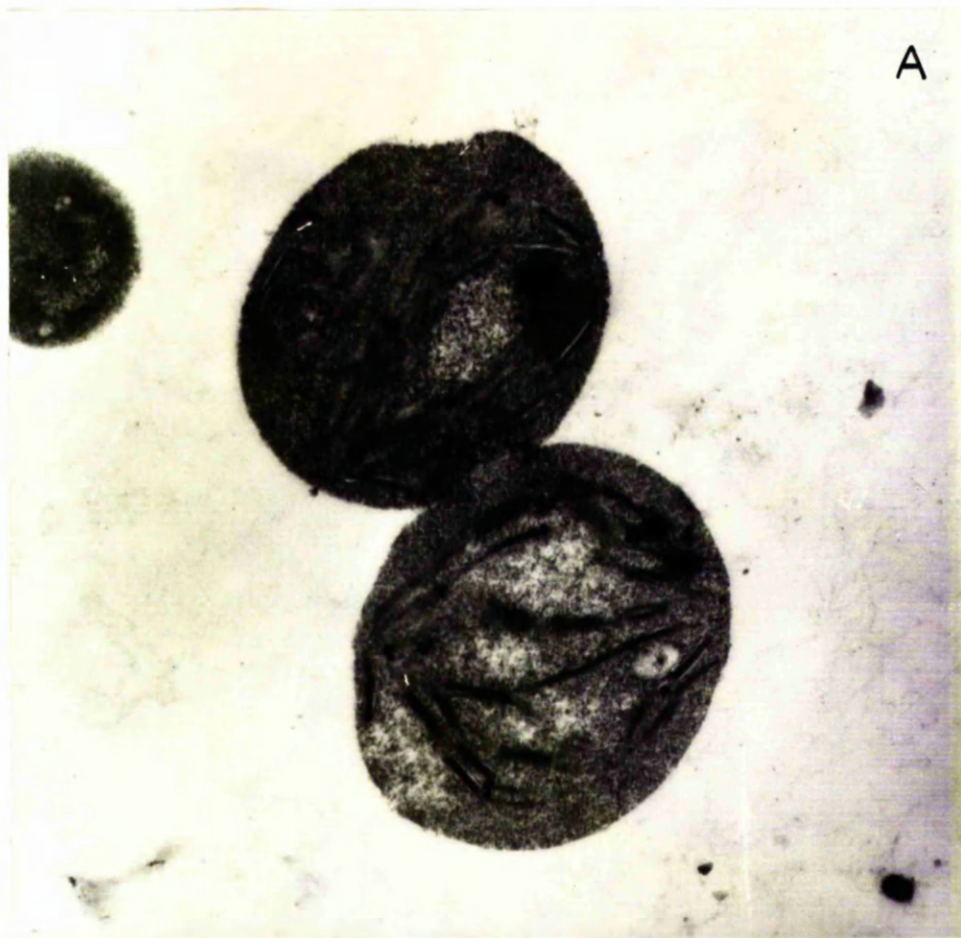
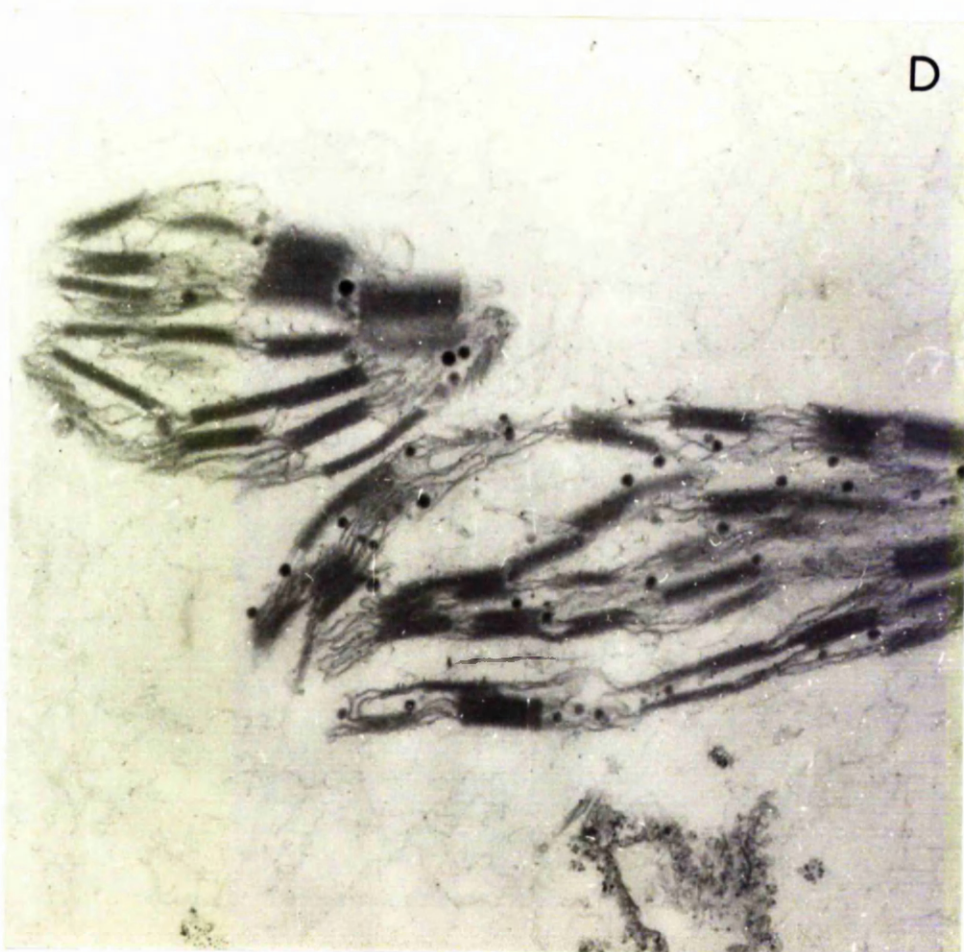
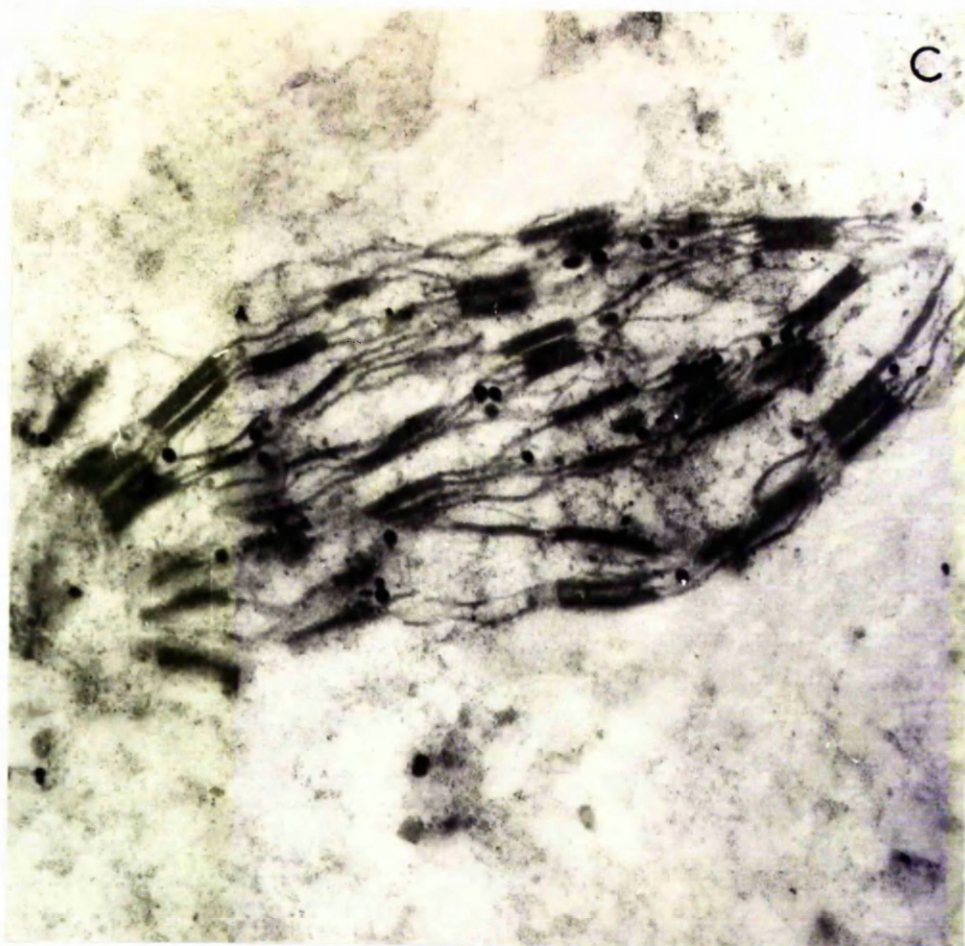


Figure 43. Electron micrographs of the various pea chloroplast preparations used for the flash-induced absorption change measurements in figure 42. A, intact (74%) chloroplasts after 10 seconds pre-illumination, magnification x 9 000; B, chloroplasts osmotically shocked in H₂O followed by 5 seconds pre-illumination, magnification x 19000; C, chloroplasts osmotically shocked in 10 mM MgCl₂, 1 mM TRIS, pH 8.2, followed by 5 minutes dark-adaption, magnification x 15000; D, chloroplasts osmotically shocked in 10 mM MgCl₂, 1 mM TRIS, pH 8.2, followed by 5 seconds pre-illumination, magnification x 15000.





3.1.11.5 The Effect of Different Cations during Osmotic Shocking on the Slow Phase in Chloroplasts

To investigate further the role of Mg^{2+} in retaining the slow phase during osmotic rupture it was decided to observe the absorption changes after osmotic shock in different cations. If the effect was specifically due to Mg^{2+} , only the traces of chloroplasts shocked in that ion should retain the slow phase. If, on the other hand, the effect is a general cation effect as found for salt-induced changes in fluorescence (Barber, 1976) then both divalent cations and monovalent cations at higher concentrations would be expected to retain some slow phase activity. Figure 44 shows the effect of various divalent cations (10mM) and monovalent cations (200mM) on the kinetics of the absorption change at 515nm. A control treatment of osmotic shocking in H_2O alone is included. The data show that all the divalent cations show the capacity to induce retention of the slow phase by varying amounts, whereas the monovalent cations do not exhibit a similar effect. This is surprising since if the effect is due to the influence of cations then all cations, not just divalent ones, should induce similar effects although since monovalent cations only carry one positive charge then higher concentrations would be necessary for the equivalent effects seen with divalent cations. It is suggested that the effect of shocking chloroplasts in Mg^{2+} is not specific for that cation anyway since Ca^{2+} and Mn^{2+} also effect a retention of slow phase during osmotic shocking.

The explanation for the lack of similar results with monovalent cations might be that the high concentrations used provide an increasing amount of ions which are able to equilibrate across the thylakoid membrane. This in turn leads to an increased rate of decay (Junge and Witt, 1968) which might prevent the slower phase of absorption increase from being observed (Farineau et al. 1980).

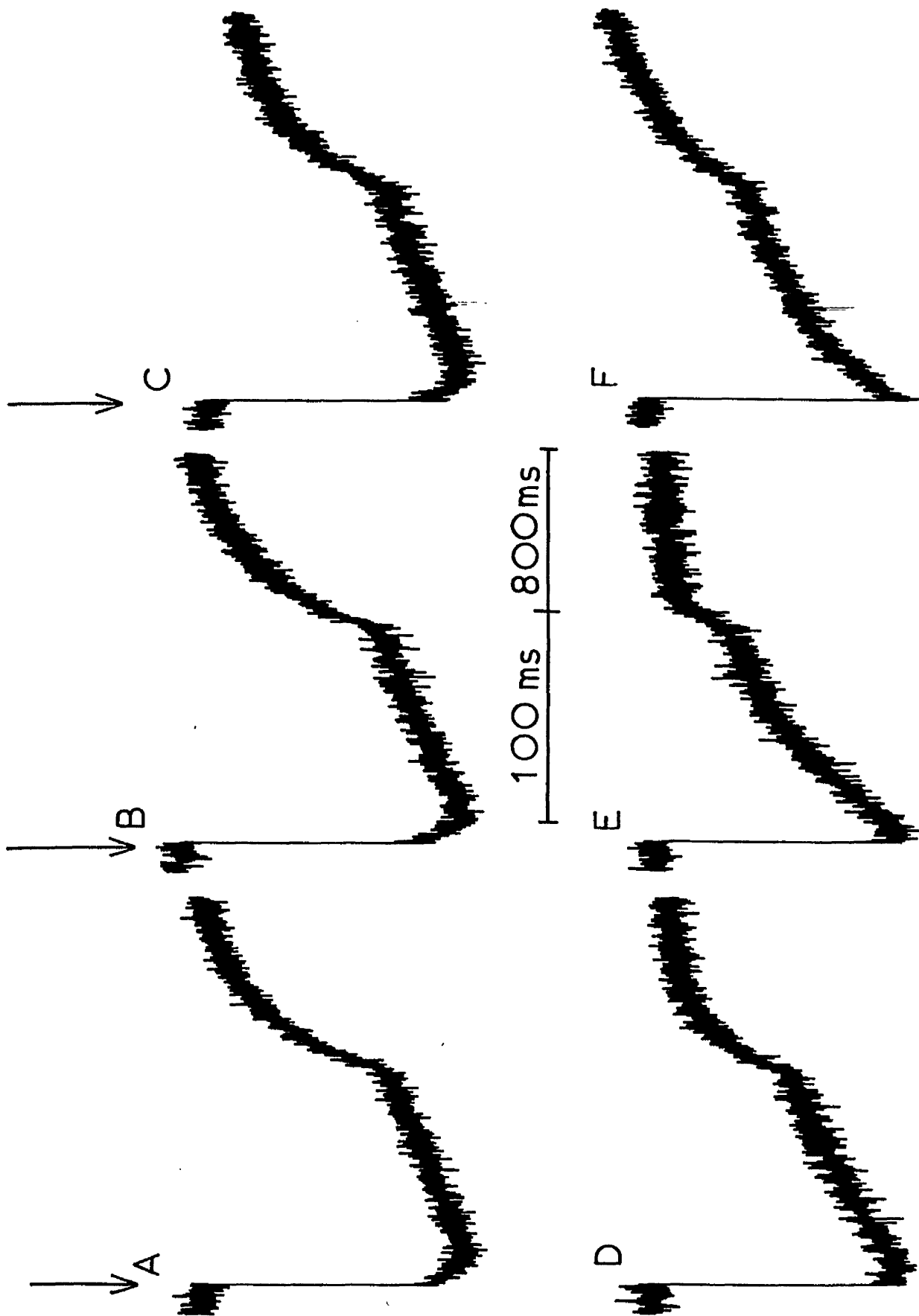
Figure 44. Typical examples of the flash-induced absorption change at 515 nm in pea chloroplasts, osmotically shocked in the presence of various cations, and pre-illuminated for 5 seconds (white light giving an intensity of 20 Wm^{-2}). A, osmotically shocked in 10 mM MgCl_2 ; B, osmotically shocked in 10 mM CaCl_2 ; C, osmotically shocked in 10 mM MnCl_2 ; D, osmotically shocked in 200 mM NaCl ; E, osmotically shocked in 200 mM KCl ; F, osmotically shocked in H_2O only. Each trace represents the accumulated average of 32 sweeps at a flash frequency of 0.5 Hz.

The transient recorder was used in the split time base mode (200msec/2sec)

The flash was fired at the point indicated by the arrow.

$\Delta I/I$
 1×10^{-3}

100 ms 800 ms



Another possible explanation is that the cations induce microscopic changes in the thylakoid membrane as shown in fluorescence and light scattering studies (Murakami and Packer, 1970; Seely, 1973). These studies have shown that cations induce changes in membrane thickness and the distance between the various pigment systems. Such changes might affect the ability of the shocked chloroplasts to show slow phase.

3.1.11.6 The Effect of CCCP Concentration on Chloroplasts Shocked in 10mM MgCl₂

It was hoped that chloroplasts osmotically shocked in 10mM MgCl₂ and thus retaining the slow phase of absorption increase might yet provide an in vitro system eliciting the stimulatory effect with low uncoupler concentrations without the problems of permeability encountered in Chlorella. Thus the effect of varying CCCP concentrations was investigated in shocked chloroplasts under both pre-illuminated (10 seconds, white light, intensity 20 Wm⁻²) and non pre-illuminated conditions. Figures 45 and 46 show however that CCCP has exactly the same effect in shocked chloroplasts as it does in intact chloroplasts and bacterial chromatophores, namely to decrease the extents of both the fast and slow phase of absorption increase and to increase the rate of decay. Thus CCCP acts only as a classical uncoupler in shocked chloroplasts, even in the presence of Mg²⁺.

3.2 Discussion

The experiments in this chapter were designed to investigate the conditions under which the slow phase of absorption increase at 515nm appears and to correlate such conditions with known processes within the light reactions of photosynthesis so that further information on the mechanism responsible for the slow phase might be gained.

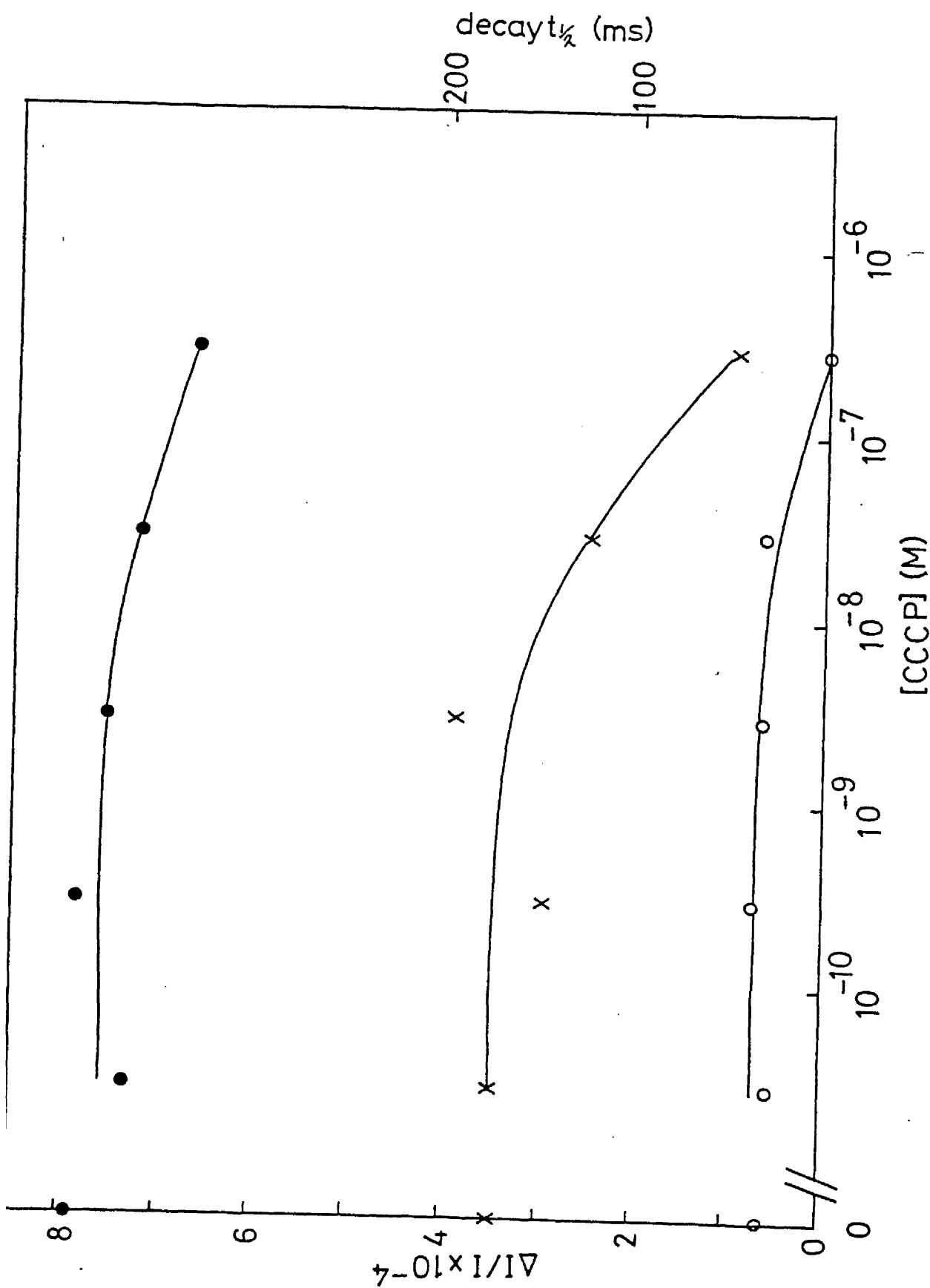


Figure 45. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in pea chloroplasts, osmotically shocked in 10 mM MgCl_2 , 1 mM TRIS, pH 8.2, and pre-illuminated for 5 seconds (white light giving an intensity of 20 Wm^{-2}). Each point represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz.

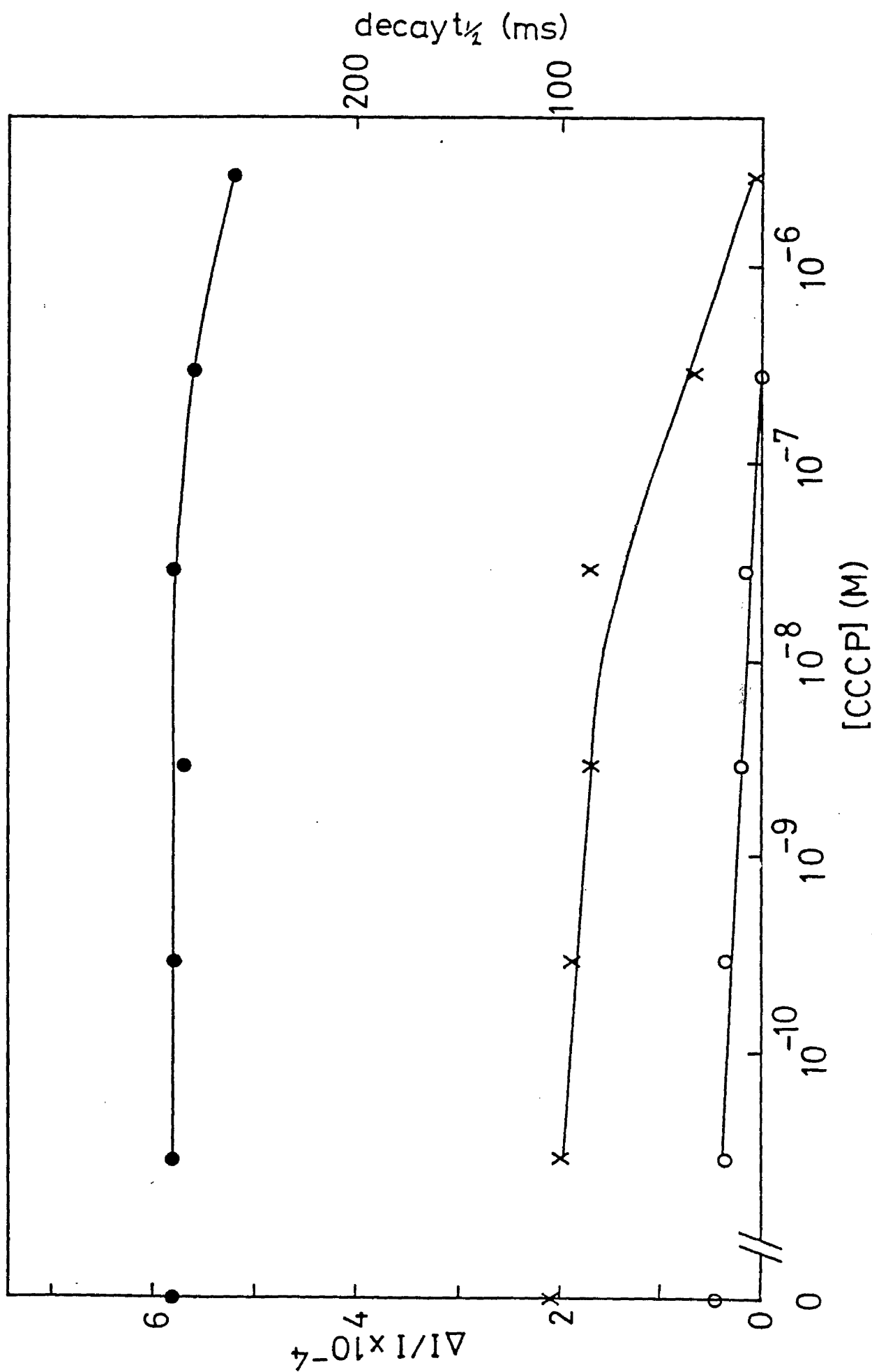


Figure 46. The effect of increasing concentrations of CCCP on the fast phase (●), slow phase (○) and decay half-time (x) of the flash-induced absorption change at 515 nm in non pre-illuminated pea chloroplasts, osmotically shocked in 10 mM MgCl₂, 1 mM TRIS, pH 8.2. Each point represents the accumulated average of 64 sweeps at a flash frequency of 0.5 Hz.

The 515 absorption change is thought to represent an electrochromic response by pigments in the photosynthetic membrane to an electric field across that membrane (see section 1.5.3) (Junge, 1977) and during the time these experiments were being conducted much interest has been shown in the unknown mechanism responsible for the slow phase of absorption increase. Such interest has been shown by researchers in photosynthesis because knowledge of the mechanism of the slow phase may lead to further information on the arrangement of electron carriers which take part in cyclic and non-cyclic electron flow in the thylakoid membrane. Our understanding of the form and functions of the electron transport chain in chloroplasts is still incomplete at present and it is hoped that the studies conducted here have lent some further understanding to the process.

It has been found that low concentrations of the classical uncouplers CCCP, FCCP and DNP all cause a stimulation of the slow phase of absorption increase at 515nm and a marked decrease in the rate of decay of Chlorella (Figures 20(A), 22(A) and 23(A)). This effect was not observed in any other photosynthetic system, but other authors have reported a similar effect in bacterial chromatophores (Amesz and Vredenburg, 1966; Jackson and Crofts, 1971) and in intact chloroplasts (Slovacek et al. 1979). Such observations are contrary to the expected effects of phenolic uncouplers (Figure 19(A), section 1.4, Junge and Witt, 1968). A second important difference between the algal system and higher plant chloroplasts was the necessity for dark adaption in the former case and pre-illumination in the latter to obtain slow phase.

Thus in Chlorella low uncoupler concentrations have the property of accelerating the dark adaption process which itself brings about the slow phase. Furthermore, saturating steady state light and increasing numbers of flashes have been found to inhibit the slow phase. Before discussing these differences in slow phase between the two systems, the possible mechanisms for the unexpected effects of the low uncoupler concentrations, and the further ideas on the actual mechanism for the slow phase which these studies have revealed one major assumption must be mentioned. It is that the slow phase of absorption at 515nm represents a third electrogenic step across the thylakoid membrane. This is concluded from the data obtained in Figure 24, the light minus dark difference spectra, which indicate that the slow phase under control conditions or when stimulated by 0.8 μ M CCCP has the same spectral characteristics as the electrogenic fast phase, and does therefore represent a third electrogenic step.

To explain the differences between the various systems it is necessary to consider what change may occur in the electron transport chain as a result of pre-illumination and dark adaption. It is generally accepted that for maximal slow phase to be present in any photosynthetic system capable of eliciting the slow phase correct redox poising is of paramount importance (Crowther et al. 1979; Jackson and Dutton, 1973). In chloroplasts this "poising" takes the form of pre-reduction of the intersystem plastoquinone pool before flash measurements are made (Velthuys, 1978). The results of Figure 39(A) agree that pre-reduction of a component by pre-illumination is a necessary prerequisite for the slow phase in chloroplasts and that too much or too little pre-illumination decreases it, suggesting that the component may become too little or overreduced depending on the length of pre-illumination.

Crowther et al. (1979) have shown that varying amounts of DCMU may be used to vary the electron input from PSII to the intersystem pool and hence the redox state of the plastoquinone pool. They suggest, in accordance with others (Velthuys, 1978) that achieving a sufficiently negative redox poise (also by addition of dithionite to intact chloroplasts) leads to an appreciable fraction of the plastoquinone pool becoming reduced and therefore optimal conditions for the slow phase are achieved. Chlorella, however does not show the same light requirements for slow phase. Figure 30 suggests that pre-illumination is actually inhibitory to the slow phase and that increasing dark adaption brings about an increasingly favourable condition for it. The decreasing amount of slow phase with flash number in Figure 31 also suggests this. Bourges-Bocquet (1980) has shown that after dark adaption the plastoquinone pool in Chlorella is only about 12% reduced whereas the slow phase is maximal. Furthermore the addition of DCMU and NH_2OH (Figure 29 and Table 1) only affects the fast phase of absorption increase in Chlorella unlike the poisoning effect reported by Crowther et al. (1979) in chloroplasts. However, the slow phase in Chlorella is suppressed by the addition of an oxidant and restored by the addition of a reductant (Bourges-Bocquet, 1977) suggesting that like chloroplasts a component must be reduced before the slow phase occurs. Also although the plastoquinone pool may not be the reduced component necessary for the appearance of the slow phase it must be very closely linked with the slow phase as the quinone antagonist DBMIB inhibits the slow phase in both Chlorella (Bourges Bocquet, 1977b) and intact chloroplasts (Slovacek et al. 1979). To allow for these differences in redox properties of plastoquinone a number of chemically uncharacterised components have been put forward as the unknown component which has to be reduced for the slow phase to occur; e.g. V (Crowther and Hind, 1980) and U (Bourges Bocquet, 1980). The unknown reduced component has been redox titrated by Crowther and Hind (1980) by titrating the appearance of the slow phase in chloroplasts and has a midpoint redox potential of -55mV (pH 8.1).

The difference between chloroplasts and Chlorella in their respective light requirements for the slow phase may be explained as follows. Assuming that the components which make up the electron transfer chain in the two systems are similar it is possible that the resting redox potential in the chloroplasts of Chlorella differs from that of the isolated higher plant chloroplasts and that they therefore require different light pre-treatments to poise the electron transport pathway engaged in the slow electrogenic step. Such a difference between the two systems is quite plausible; as the algal chloroplast is surrounded by cytoplasm and will thus be differentially buffered against external changes in pH, oxygen and CO₂ tension. The isolated chloroplasts, both intact and shocked are suspended in an artificial medium, the conditions of which are probably different from most of the cytoplasm surrounding the algal chloroplasts, and it is well-known that carbon fixation rates are governed by the pH and CO₂ and oxygen tensions of the stroma (Berry and Farquar, 1978; Heldt et al. 1978). By altering the rates of carbon fixation, changes in the electron transport will be induced. It is suggested that isolated chloroplasts would be more greatly exposed to such changes than would the chloroplasts in intact algal cells.

The stimulatory effect on the slow phase of absorption increase by low uncoupler concentrations in Chlorella may be seen as an acceleration of the dark adaption process and thus may also be explained by their influence on the redox state of the electron transport components of the algal chloroplast. Electron flow in the thylakoid is thought to be regulated by the magnitude of the transmembrane electrochemical gradient. According to the chemiosmotic theory, the major reason for this is thought to be that as electron transport drives protons across the thylakoid to the inner space a pH concentration gradient is built up across that membrane.

As this pH gradient becomes larger so a back pressure will build up. Because the proton pumping is coupled to electron transport, the rate of electron transport will be decreased, i.e. the rate of re-oxidation of plastoquinol and consequently (see section 1.3.3) the rates of reduction of cytochrome f and plastocyanin would be directly related to the proton concentration of the inner bulk phase (Avron, 1978). Secondly, it has been reported that the apparent midpoint redox potentials of electron transport components in mitochondrial, bacterial and chloroplast membranes are influenced by the electric field across that membrane (Hinkle and Mitchell, 1970; Takamiya and Dutton, 1977; Bohme and Cramer, 1973; Mitchell, 1968). The mechanism for this dependence is unknown but it may be due to changes in the dielectric constant in the neighbourhood of the heme groups of the cytochromes. If the ferric form of the cytochrome had a larger absolute charge in the neighbourhood of the heme iron the effect of an increase in dielectric constant by the presence of phenolic uncouplers will be to lower the cytochrome midpoint potential, since the ferric form would be stabilised (Bohme and Cramer, 1973). Low concentrations of uncoupler may therefore induce enough change in the trans-thylakoid electrochemical gradient to alter either the redox poise of the components of the electron chain which bring about the extra electrogenic step, i.e. cytochrome b₅₆₃, cytochrome f etc., or the rate of electron transport through them by decreasing the back pressure that the electrochemical gradient exerts on the electron transport chain. Increasing concentrations of uncoupler would increase the rate of decay of the absorption change to the extent that the slow phase would be prevented from being seen.

This hiding of the slow phase leads to another possible mechanism by which the low uncoupler concentrations might stimulate the extent of the slow phase. It has been suggested by Farineau et al. (1980) that the 515 absorption change is of a composite character being made up of at least two exponential curves. These kinetic components may be separated by means of a stepwise deconvolution to a linear combination of the various exponentials. The slow phase of absorption increase may be present all the time but if the decay of the overall absorption change is too fast then the slow increase will be hidden. Garab (personal communication) has suggested that the reasons for the slow phase being observed is that the rate of decay is decelerated as can be shown by deconvolution. The data of Tables 2 and 3 were derived by deconvoluting absorption increases in the presence of low and high uncoupler concentrations and concentrations of KCN and HgCl_2 which stimulate the slow phase and with no addition and the results would seem to concur with Garab's suggestion; in all cases of stimulation of the slow phase in Chlorella the rate of decay is decreased. Therefore a second possible mechanism for the slow phase stimulation by the low uncoupler concentrations is that the slow phase is present all the time but that the decay of the overall change under phosphorylating conditions is too fast for the slow phase to be seen. If the decay is slowed down by the low uncoupler concentrations, as it generally is in Chlorella when the slow phase is stimulated, then the slow phase will become more apparent.

The absence of any stimulation of slow phase in intact and broken chloroplasts and the bacterial photosynthetic systems suggests that either their electron transport components which are responsible for the slow electrogenic step are already correctly poised or that the mechanism by which an uncoupler could poise a system is lost during the isolation of in vitro photosynthetic systems, i.e. the uncoupler may exert its effect in whole algae by slightly increasing the ionic permeability of the thylakoid membrane or chloroplast envelope whereas the corresponding membranes in

isolated systems may already have become significantly permeable during the isolation procedure. As in the explanation for the different light pre-treatments necessary for maximal slow phase in chloroplasts and algae differences in the exposure of the photosynthetic membranes to external changes in pH or oxygen and CO₂ tensions may also be responsible for the mechanism by which low uncoupler concentrations stimulate slow phase being lost in in vitro systems.

The stimulatory effect found with KCN in Chlorella is unexpected as one would presume that if this compound blocks electron donation to PSI at the site of plastocyanin (Ouitrakul and Izawa, 1973) then its effect would be to inhibit the extent of the fast and slow phases. It has been shown however that it causes a stimulation of the slow phase and a marked decrease in the rate of decay over the concentration range in which it begins to inhibit oxygen evolution in a similar way to CCCP and the other phenolic uncouplers. An early report by Slater (1955) did suggest that cyanide does in fact uncouple at high concentrations. This has prompted a further possible mechanism for the stimulation of the slow phase. It is suggested (R. Cogdell personal communication) that KCN acts in this unusual way by dissociating into K⁺ and CN⁻ ions. The CN⁻ ions may pass across the thylakoid membrane and become protonated inside to form HCN (pK_a = 9.31). This would result in the dissipation of the pH component of the electrochemical gradient (ΔpH) without any change in the electrical component ($\Delta\psi$). On loss of the pH component, the electrogenic proton pump would be expected to generate a compensating increase in the electrical component. Thus the increase of the slow phase of the 515 absorption change would be attributed to an increased membrane potential which arises to compensate for the loss of the pH component of the proton gradient across the thylakoid membrane.

To test this theory it was hoped that the action of NH_4Cl , would provide further evidence as it is thought to uncouple by dissipating the pH component and not the electric field of the high energy state, as in the mechanism above (McCarty, 1969). However, no effect, either on oxygen evolution or the absorption change was found to be induced by concentrations as high as $3.3 \times 10^{-2}\text{M}$ and it was presumed that the compound did not penetrate the algal chloroplast. It should be noted that the compound is reported to stimulate the slow phase in isolated chloroplasts by Slovacek et al. (1979); they propose that this is associated with rapid electron transfer through cytochrome b₅₆₃, plastoquinone and cytochrome f as is the stimulatory effect on the slow phase induced by other uncouplers, CCCP and Nigericin.

The mechanism suggested for KCN does not explain the results obtained with HgCl_2 , which induces similar results but is not thought to dissociate to form a weak acid as KCN may. Like KCN it is reported to block plastocyanin (Kimimura and Katoh, 1972) and its decreasing effect on the extent of the fast phase may be assumed to concur with this role in blocking the light reactions at one of the photosystems. However, its stimulation of the slow phase and marked decrease in the rate of decay at 10^{-4}M followed by an increasing rate of decay as the concentration is increased suggest that it is acting in an uncoupling manner. The effect of Hg^{2+} is considered in more detail in the chapter on heavy metals.

The experimental strategy employed to determine which part of the photosynthetic process was responsible for the slow phase of the electrochromic band shift was to study different segments of the light reactions in Chlorella under conditions where the slow phase is maximal by addition of $0.8\mu\text{M}$ CCCP and under control conditions.

Thus the effects of CCCP concentration were studied on oxygen evolution, PSII chlorophyll fluorescence, the 515 absorption change with PSII blocked by DCMU and NH_2OH and the redox changes of cytochrome f and b_{563} . The data obtained with oxygen evolution suggested that concentrations of CCCP which stimulate the slow phase have no effect and it is concluded therefore that the slow phase is not directly correlated with changes in the overall rates of steady state linear electron transport from H_2O to the dark carbon fixation cycle. Both the studies on PSII chlorophyll fluorescence and blocking photosystem II with DCMU and NH_2OH indicate that the slow phase is not directly associated with PSII. This concurs with the idea that the slow phase is associated with PSI and the flow of electrons around it. The data on the effect of varying CCCP concentrations on the redox changes of cytochrome f and b_{563} should help to determine which segment of the electron transport chain around PSI is responsible for the slow phase. As was discussed in section 1.3.3 and 1.5.3.5.1 the intersystem electron transport chain may accept electrons from either the acceptors of PSII or from cyclic flow from the electron acceptors of PSI, primarily ferredoxin. Such a cyclic system may include the electron carrier cytochrome b_{563} . Therefore if the slow phase was associated with the intersystem electron transfer chain then it should be possible to correlate the rates of electron flow through cytochrome f and the size of the slow phase. Furthermore if the slow phase is also associated with some type of system, be it cyclic or a Q cycle type mechanism involving cytochrome b_{563} , its kinetics should also correlate with the size of the slow phase. Neither stimulating the slow phase with CCCP or KCN induced a concurrent change in cytochrome b_{563} kinetics which does not generally agree with the literature (see Malkin, 1982; Cox and Olsen, 1982),

nor does the decrease in reduction rates of cytochrome f associated with the stimulation of the slow phase by CCCP and KCN. The increasing kinetic rates of both cytochromes in the presence of strong uncoupling concentrations is however in keeping with the literature and the accelerated turnover times in the presence of the uncoupler indicate the existence of a coupling site between cytochrome b₅₆₃ and cytochrome f. A possible explanation for the discrepancy between the reported behaviour of cytochrome b₅₆₃ and its kinetics here when the slow phase is stimulated may be obtained if the assumption, of low uncoupler concentrations not effecting the slow phase but just decreasing the rate of decay so that the slow phase is more easily detected, is made. In this case the slow phase is not actually changing and so the kinetics of the cytochrome might also not be expected to change. I have no explanation however for the decreasing kinetic rates for cytochrome f unless the section of the electron transport chain responsible for the slow phase bypasses this carrier, possibly via the other carrier known to be at the same redox potential, i.e. plastocyanin. An early hypothetical mechanism for the slow phase by Bourges-Bocquet (1977b) did suggest that the slow phase was correlated with plastocyanin reduction and a number of reporters have suggested a parallel rather than linear electron flow from PQ through cytochrome f and plastocyanin to P700 (see section 1.3.3.3). The cytochrome f data may thus be explained as an increased rate of electron flow through plastocyanin to P700 rather than an increased rate through cytochrome f.

As mentioned earlier a possible mechanism for the stimulation of the slow phase might be changes in the redox potentials of the carriers responsible for generating the slow electrogenic step.

This theory may be applied to cytochrome f; Cramer et al. (1974) found that low ($1-4\mu\text{M}$) concentrations of FCCP which did not affect steady state oxygen evolution interfered with the chemical oxidation and reduction of cytochrome f and suggested that this was caused by structural changes in the membrane which may be a side effect of the carbonyl cyanide type uncouplers. Therefore, although the cytochrome changes must be treated with caution by the nature of their derivation, it is possible to correlate the increased slow phase with a decreased flow of electrons through cytochrome f by suggesting it lies on a side pathway (Bourges-Becquet, 1977b).

The decrease in the rate of decay caused by low concentrations of uncouplers, KCN and HgCl_2 as well as dark adaption in Chlorella, is obviously linked in with the stimulation of the slow phase as can be seen by the similar concentration dependence. As has already been discussed, this decrease in decay rate may possibly be the cause of the stimulation itself. However, the fact remain that the major property of uncouplers should be to increase the rate of decay of the absorption change by increasing the ionic permeability of the thylakoid. How then may uncouplers, albeit at low concentrations, lead to a decreased rate of decay? It is accepted that a decreased rate of decay of the 515 absorption change results from a decreased flux of ions across the membrane either positively outwards or negatively inwards (Junge and Witt, 1968). One of the major contributions to the decay is evidently from outward movement of protons via the coupling factor during phosphorylation (section 1.4) and if this movement of protons is prevented, either by chemically blocking the coupling factor with an inhibitor such as DCCD (McCarty, 1980) or by using mutants blocked at the terminal stage of phosphorylation, such as Chlamydomonas F54 (Joliot and Delosme, 1974) then a decreasing rate of decay with decreasing phosphorylation activity is observed (Rumberg and Siggel, 1968; Junge and Witt, 1968; Joliot and Delosme, 1974).

Therefore it is possible that the decrease in decay rate caused by low uncoupler concentrations and dark adaption represent a lack of outward proton movement during phosphorylation. A further possible clue to this mechanism is that both dark adaption and the low uncoupler concentrations essentially have the same effect. Thus the low uncoupler concentrations may just be increasing the rate of the dark adaption process which prevents phosphorylation. It has been shown by several workers that photophosphorylation in chloroplasts is preceded by a lag phase when phosphorylation is induced by light flashes, acid base transitions or continuous light (Harris and Crofts, 1978; Junge et al. 1970; Smith et al. 1976; Ort and Dilley, 1976), This lag phase which occurs before phosphorylation can proceed, has been recently correlated with a number of processes thought to be involved in a conformational activation of the coupling factor, and not with the build up of the critical proton concentration (see Schlodder et al. 1982). Thus even a high electrochemical gradient across the thylakoid will not lead to phosphorylation unless the coupling factor is activated. The activation itself may however be caused by the steady state build up of the membrane potential or proton gradient under conditions not yet fully understood (Morita et al. 1981) or by artificial activation such as an electron donating system involving thioredoxin suggested by Mills et al. (1980). Various observations of this activation have been shown recently (see review by McCarty, 1979; Schlodder et al. 1982). One possible mechanism is that proposed by Graber et al. (1977) and Harris and Crofts (1978) who proposed that a conformational change of the ATPase, induced by ΔpH or $\Delta \psi$ removes the inhibitory ϵ -subunit from its inhibiting site thus letting H^+ move through the CFo segment of the coupling factor.

A second observation is that the CF₁ contains tightly bound adenine nucleotides. (AMP, ADP and ATP). Energization of the membrane and activation of the coupling factor is correlated with a rapid exchange of these nucleotides (Schlodder and Witt, 1981). The function of these nucleotides in the mechanism is still a matter for discussion at present but it is widely accepted that the exchange shows that activation of the complex does involve conformational changes. The activation is induced by light and its rate of induction dependent on the energy input to the electron transport chain such that increased flash frequency shortens the activation time whereas addition of DCMU lengthens it and a larger number of flashes were needed to induce activation (Harris and Crofts, 1978). Furthermore not all the coupling factors are thought to be activated at the same time and thus the rate of H⁺ efflux and phosphorylation will be governed by the ratio of activated to unactivated complexes, providing a large enough electrochemical gradient across the thylakoid exists (Harris and Crofts, 1978). The activation has also been shown to be inhibited by uncouplers of photo-phosphorylation (McCarty, 1979). Thus treatments which prolong the period of activation would be expected to prolong the time preceding phosphorylation and therefore the increased rate of decay of the 515 absorption change which is associated with the outward flux of protons. At least two reports have concurred with this proposal, Morita et al. (1981) showed that the dark decay of the 515 absorption change in maize leaves was reversibly accelerated by pre-illumination. The acceleration was not observed when the CF₁ was already activated by the high energy state as shown by fluorescence studies indicating that the acceleration was related to the formation of the high energy state by pre-illumination. They proposed that pre-illumination induced structural changes in CF₁ by way of the high energy state. These structural changes in CF₁ led to an increased permeability of the CF₁ to protons.

They further suggested that deactivation of the CF₁ proceeded very slowly in intact leaves resulting in a long dark adaption being necessary before the rate of decay of the absorption change returned to the initial slow rate. This agrees with the results found in Chlorella here. As the effect of low uncoupler concentrations here is to decrease the rate of decay in a manner similar to the dark adaption; it is suggested that low uncoupler concentrations prevent the activation of the coupling factor (as reported by McCarty, 1979). It is presumed that these concentrations are not high enough to render the membrane highly permeable to protons as is seen at higher concentrations (Figures 20(A), 22(A) and 23(A)) and thus no increase in the rate of decay is caused by protons leaking back across the membrane via the uncoupler.

The final point of discussion in this section is regarding the appearance of the slow phase seen when chloroplasts are shocked in Mg²⁺ and the other divalent cations. It was suggested (Shahak et al. 1980) that this results from the necessity of Mg²⁺ for the binding of ferredoxin in the thylakoid which is thought to be a component of the electron transfer chain which is responsible for the slow phase (possibly cyclic electron flow around PSI, Crowther and Hind, 1980). The results showing shocking in other divalent cations do not altogether support this concept unless the binding of ferredoxin is also induced by other cations. Had both monovalent and divalent cations induced the slow phase, an explanation may have been given that the cations induced changes in the diffuse double layer of negative charges around the thylakoid. Cations are known to influence a number of photosynthetic processes such as electron transport and chlorophyll fluorescence, possibly by varying the quantum distribution between the light harvesting matrices of the two photosystems (see Barber, 1976).

Also the cation concentration plays a major role in the stacking of the thylakoid membranes to form grana (Murakami and Packer, 1971). Furthermore Telfer et al. (1980) have shown the binding of the coupling factor in chloroplasts to the thylakoid membrane is dependent on the presence of cations probably because negative charges on the CF_1 component and its binding site are electrostatically screened by cations. They further showed that the increase in decay rate of the 515 absorption change induced by the release of the coupling factor from the membrane by EDTA is prevented by cations; the order of effectiveness being dependent on the charge of the cation. Thus the ability of cations to prevent the slow phase being lost during osmotic shocking might be due to their preventing the loss of the coupling factor and therefore retaining a rate of decay of the 515 absorption change slow enough for the slow phase to be observed. The mechanism by which cations effect such changes in thylakoid activity are complex. Mills and Barber (1978) have shown that changes in the cation concentration of the thylakoid environment can induce changes in the electrical double layer thought to exist at the interface of the outer thylakoid surface and the surrounding medium. This may result in changes in both membrane surface potential and electrical potential difference across the membrane. These changes in the charge distribution around and in the membrane are thought to produce alterations in the microconformation of the membrane and therefore affect various photosynthetic activities. Interactions between the proteins and lipids in the membrane will be affected such that the chlorophyll-protein complexes and electron carrying components may move laterally in the membrane and induce changes in the various light reactions with which the components are concerned. Since these changes are generally nonspecific for the cations employed, excepting that monovalent cations carry less charge than divalent ions and therefore greater concentrations of monovalent cations are needed for the same divalent cation effects, the differential amounts of slow phase observed when chloroplasts are shocked in monovalent and divalent cations

seems to invalidate any connections with the above mechanisms. As suggested in the results section it is possible that the higher concentrations of monovalent cations employed may induce an increased rate of ion redistribution across the membrane preventing the slow phase being seen.

It was hoped that the investigation of the slow phase and the conditions under which it appeared in Chlorella might lead to further information regarding the mechanism of its origin. However one major problem was consistently encountered in these studies; that of the inability of a large number of the compounds, which are generally used to isolate the various sections of the light reaction in chloroplasts, to freely permeate into the chloroplast and thylakoids of the algae. This was particularly unfortunate in that the major method of stimulating the slow phase by low uncoupler concentrations was found to only occur in the intact Chlorella cell and not in any of the other in vitro systems upon which, it is now concluded, it would have been much easier to investigate the detailed segments of the electron transport chain. It is suggested that studies using intact cells are far more dependent on biophysical techniques such as absorption and fluorescence spectroscopy than are the in vitro preparations of chloroplasts and chromatophores. A major technique which would have been useful for these studies but which cannot be used in intact cells is redox potentiometry by which the redox potentials of the electron transport components responsible for the slow phase might have been deduced.

4.1 Copper

4.1.1 Results

Of the four heavy metals to be studied, it was decided to study copper first. This was because most previous work had been carried out on this heavy metal and thus experimental procedures could be optimised with a prior knowledge of the concentration likely to inhibit photosynthesis in Chlorella, i.e. Cedeno-Maldonado and Swader (1974) have previously reported that copper inhibits photosynthesis in the concentration range of 10^{-4} - $10^{-3}M$ and so it was presumed that if much higher concentrations of copper were necessary to inhibit photosynthesis in these experiments then errors in the experimental procedures might be present. These errors might arise from the well known ability of heavy metals to bind to buffers (Good et al. 1966) or from their interfering with the electrochemical reactions involved in measurements of photosynthetic activity. Such problems might occur in reactions of heavy metals with the metal electrodes in the steady state and flash oxygen measuring cells (see section 1.5.1). Other forms of experimental errors might arise from (a) the formation of heavy metal precipitates which might interfere with optical measurements etc or (b) the absorption of red fluorescent light by the metal solutions themselves.

4.1.1.1 The Effect of Copper Concentrations on Oxygen Evolution

In order to investigate the effect of copper on the overall photosynthetic reaction from H_2O splitting to carbon fixation the evolution of oxygen by Chlorella, using CO_2 as the terminal electron acceptor, was studied after incubation with increasing concentrations of copper II sulphate.

Initially the same conditions as had been used for the studies on the slow phase of absorption increase were used, i.e. Chlorella at a chlorophyll concentration of $35\mu\text{g ml}^{-1}$ were resuspended in growth media containing phosphate buffer, pH 6.5 (see appendix 1). However, no inhibition of oxygen evolution was found even at concentrations as high as $3.3 \times 10^{-2}\text{M}$. It was suggested that this was due to the binding up of the copper ions by the phosphate, particularly since a precipitate became apparent at such high concentrations. Thus it was decided to use a number of different buffers for the copper incubations to determine under which conditions the copper inhibition occurred and if so which conditions afforded the lowest copper concentration of inhibition. The buffers employed were HCO_3^- , pH 7.6, Tricine, pH 7.6, TES, pH 7.5, HEPES, pH 7.0 and MOPS, pH 7.0. Only when HEPES or MOPS buffer were employed was oxygen evolution inhibited within the $10^{-4} - 10^{-3} \text{ M}$ range reported by Cedeno-Maldonado and Swader (1974) as the inhibitory concentration range, suggesting that the others bound to copper (Good and Izawa, 1972). Of the two buffers, HEPES and MOPS, it was further decided to use MOPS as HEPES has been reported to interfere with the Folin protein assay which involves copper (Good and Izawa, 1972). Furthermore, in order to determine the amount of metal binding occurring between the heavy metals and MOPS buffer, the pH of the buffer was titrated against NaOH in the absence and presence of high concentrations of the various heavy metals. From the displacements of the pKa caused by addition of the heavy metals, it was possible to obtain approximate values for the metal-buffer binding constants (K_m) according to the derivation given by Good et al. (1966) and shown in appendix 4. Figure 47 shows the pH titration curves for MOPS buffer under control conditions and in the presence of $1.7 \times 10^{-4}\text{M}$ CuSO_4 , $3.0 \times 10^{-4}\text{M}$ HgCl_2 , $5.0 \times 10^{-3}\text{M}$ $\text{Pb}(\text{NO}_3)_2$ and $2.5 \times 10^{-4}\text{M}$ ZnSO_4 at 21°C ; the log K_m values for each of the heavy metals which were derived from the pH curves were 0.480, 1.707, 0.565 and 0.447 respectively. These comparatively low values for log K_m indicate a relatively small binding of these metal cations to the buffer (Good et al. 1966).

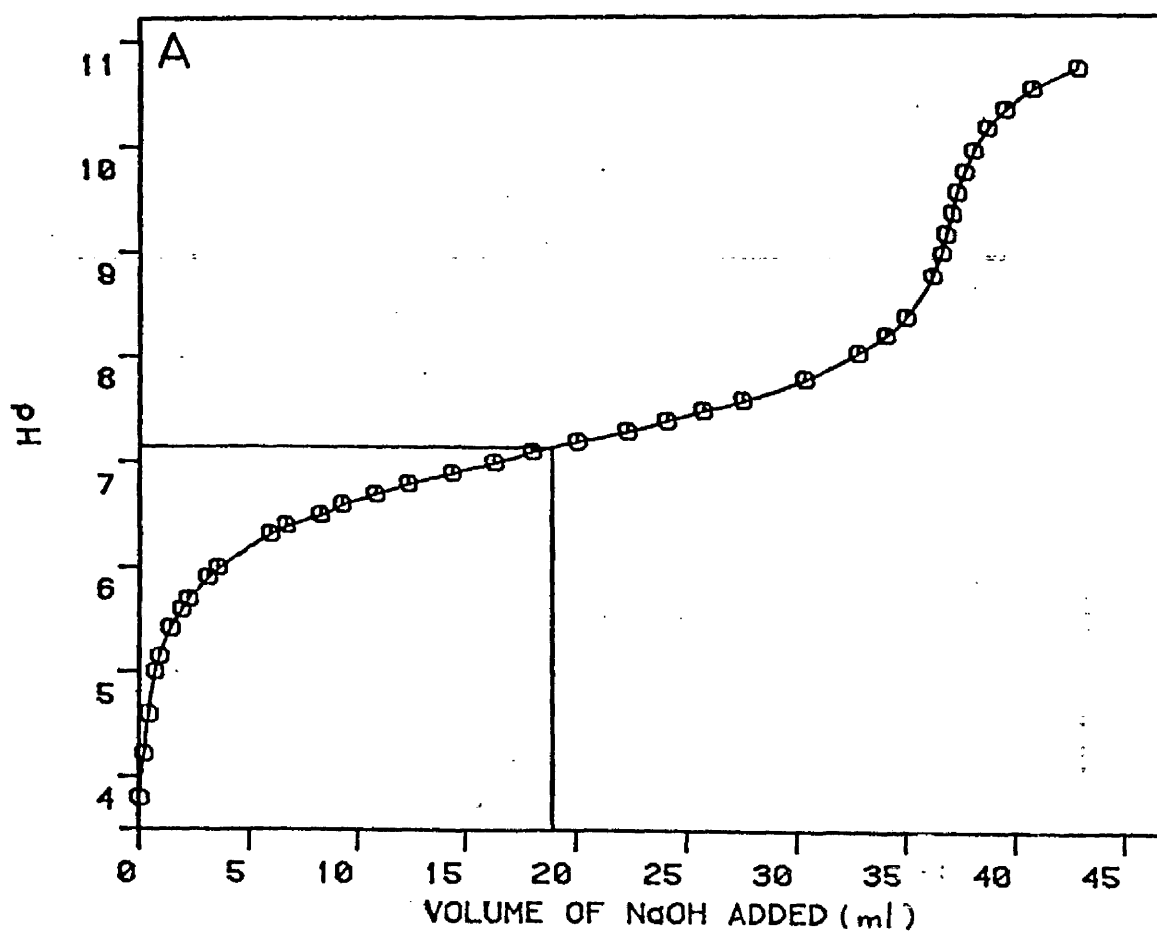
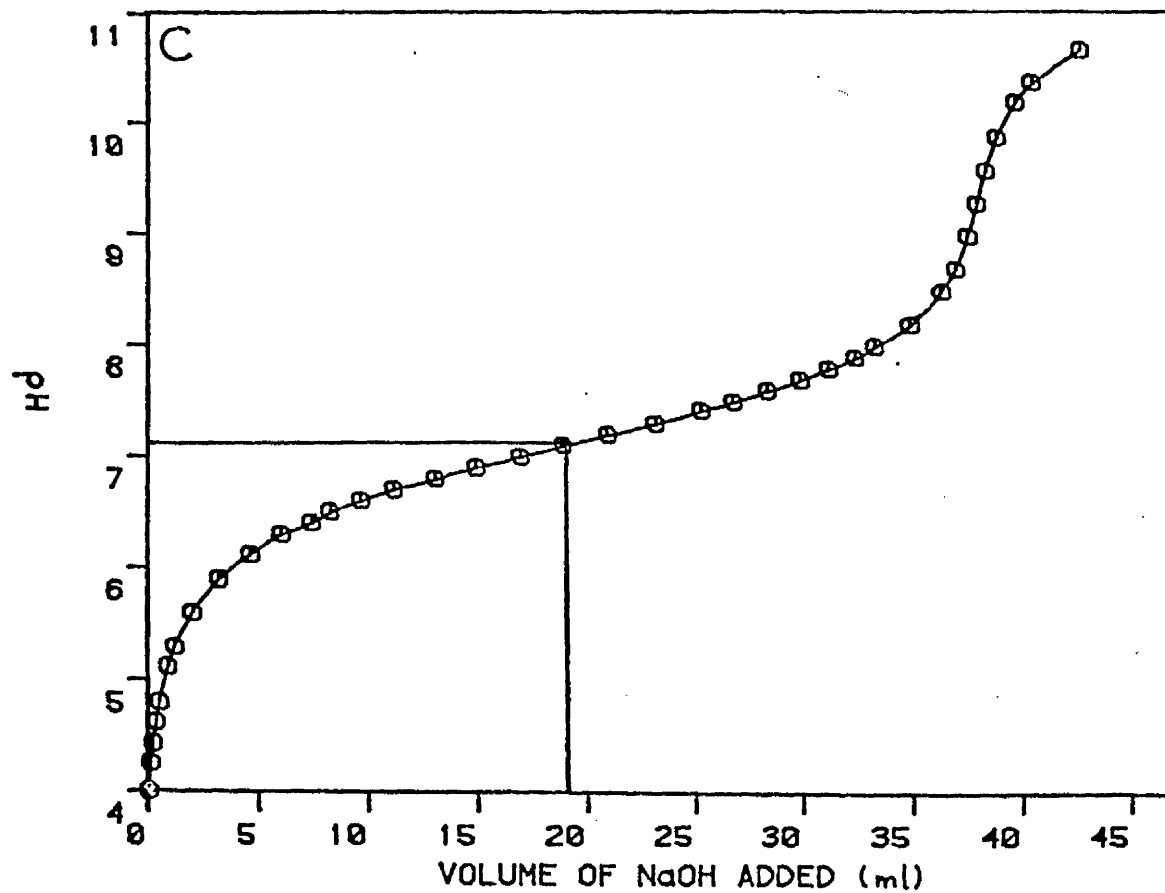
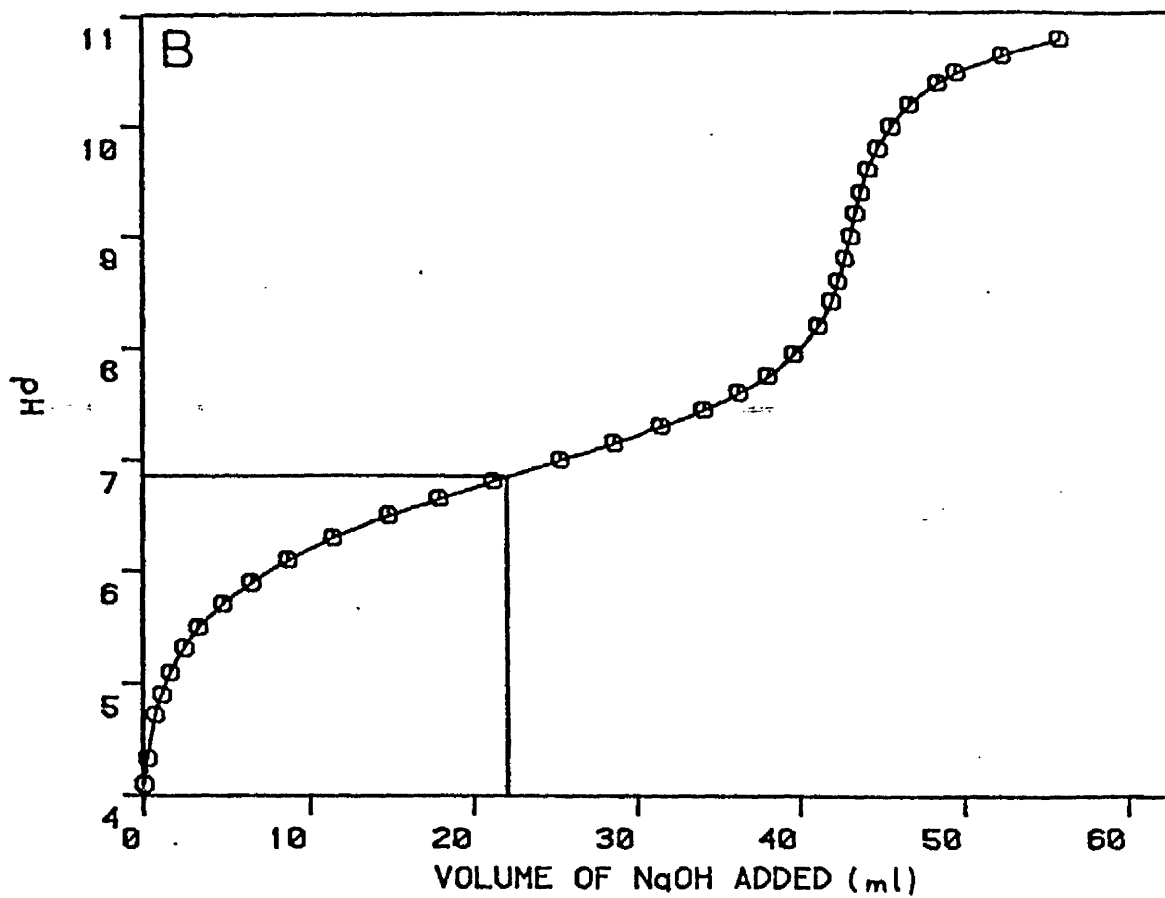
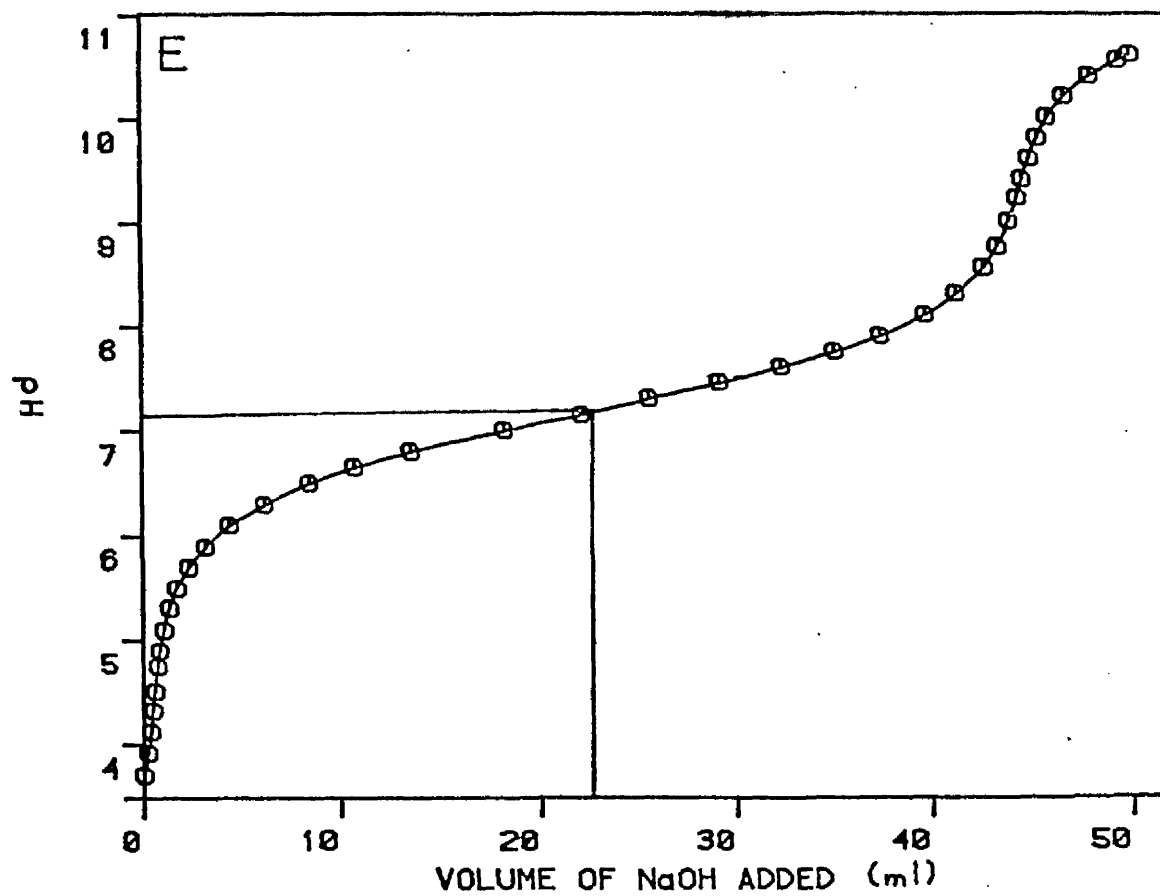
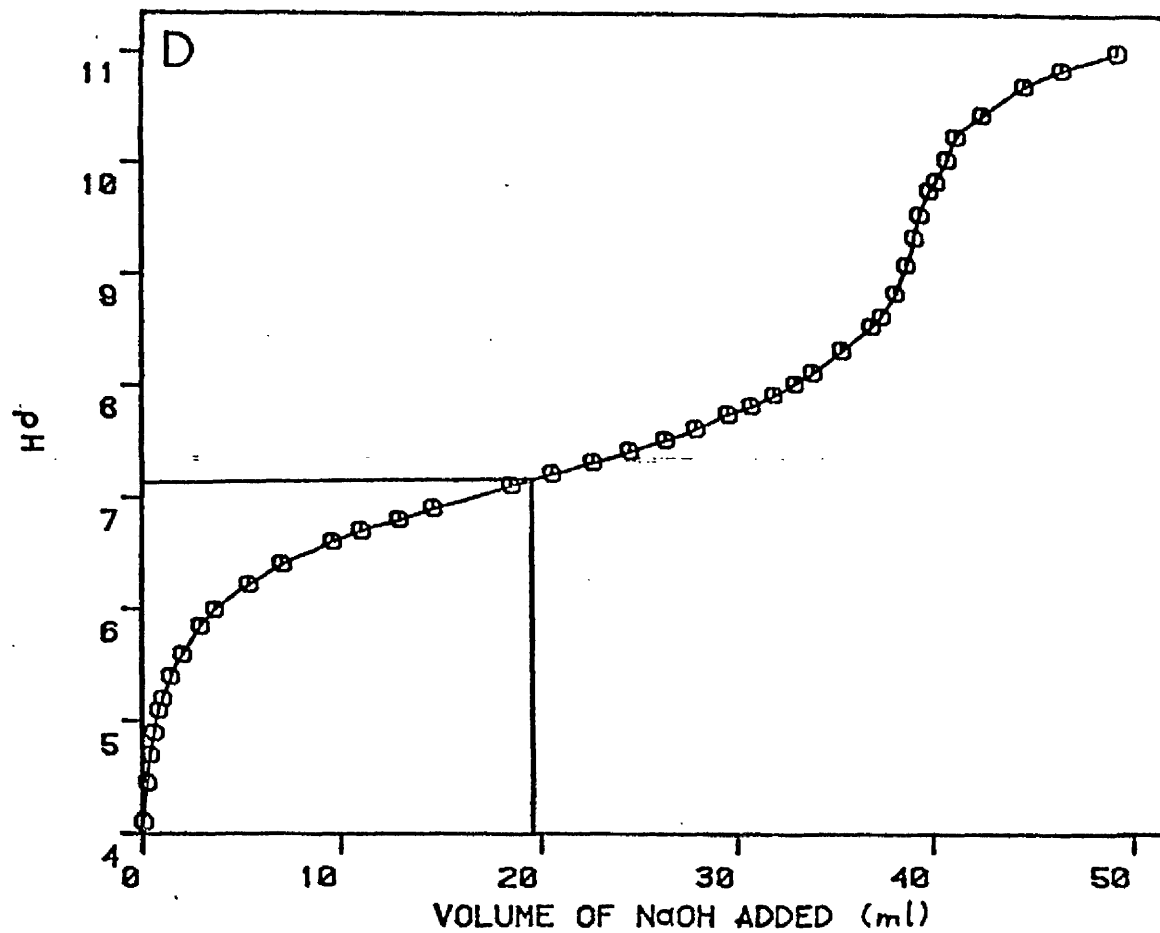


Figure 47. pH titration curves of MOPS buffer with NaOH in the presence of the various heavy metals to determine the displacement of the pH midpoint values for the derivation of the metal-buffer binding constants. Initial concentration of MOPS was 40 ml of 0.01M. Concentration of NaOH titrated was 1.15M. A, no metal addition; B, with the addition of 3.0×10^{-4} M HgCl_2 ; C, with the addition of 1.7×10^{-4} M CuSO_4 ; D, with the addition of 2.5×10^{-4} M ZnSO_4 ; E, with the addition of 5.0×10^{-3} M $\text{Pb}(\text{NO}_3)_2$.





The acid dissociation constant, K_a , of MOPS was determined from the titration curve without heavy metal addition (Lehninger, 1975; Morris, 1974). The pK_a value for MOPS was found to be circa 7.125 at 20°C in agreement with Good and Izawa (1972).

These metal buffer binding constants are only approximate; these experiments were not carried out with a view to determining accurately physical constants but rather to show that although some binding of the heavy metals to the buffer MOPS occurs, the relative amounts are small in agreement with Good and Izawa (1972); therefore, most of the heavy metal salt added is in solution and available to act on the algae in the appropriate manner.

A second problem, which was encountered, involved saturation of the oxygen electrode cell by oxygen. This resulted from the necessity of illuminating the algae for five minutes before the measurement was taken to allow a steady rate of oxygen production to develop. Therefore, to remove oxygen from the algal suspension nitrogen was bubbled through the sample before it was illuminated. The incubation therefore lasted for 10 minutes; 5 minutes in the dark followed by 5 minutes in the light before the rate of oxygen evolution was noted. Having removed oxygen from the suspension by bubbling with nitrogen it was found that the procedure also reduced the CO_2 concentration in the suspension and so 1mM $NaHCO_3$ was added before illumination but after the five minutes dark incubation to prevent the rate of photosynthesis being limited by the carbon fixation process.

The concentration of algae to be used in these experiments was determined by two experiments. Firstly the effect of varying chlorophyll concentration on the inhibition of oxygen evolution by $10^{-4}M$ $CuSO_4$ was investigated. The results are shown in Table 4.

Table 4

Effect of Chlorophyll Concentration of Chlorella on Inhibition
of O₂ Evolution by 10⁻⁴M CuSO₄ after 5 Minutes Dark Incubation

<u>[Chlorophyll] (μg ml⁻¹)</u>	<u>Inhibition (% Control)</u>
30	22.7
20	36.1
10	81.5
5	81.25

The data indicate that the amount of inhibition by copper is related to the copper to chlorophyll ratio rather than the total copper concentration in the incubation medium; the higher the amount of chlorophyll, the greater the amount of copper required to cause the same degree of inhibition. This also might suggest that the cupric ions are bound by the algal cells and would thus remove the total copper ion concentration available to inhibit photosynthesis. Similar conclusions have been drawn by Cedeno-Maldonado et al. (1972) using isolated spinach chloroplasts and Steeman-Nielsen et al. (1969) who observed a decrease of copper toxicity in Chlorella by high cell concentrations and suggested that this was caused by binding of cupric ions to cell walls and slime envelopes.

Secondly the slight binding of copper ions to the MOPS buffer as suggested by the pH titration curves, caused a precipitate to form at high copper concentrations. Such a precipitate might be expected to cause errors in the fluorescence measurements which were undertaken (see sections 4.1.1.3 and 4.1.1.4) by reabsorbing emitted fluorescence. Thus the effect of adding increasing concentrations of CuSO_4 to MOPS buffer was studied on the transmission of 695nm light through the solution. 695nm light was chosen for this experiment because it is the wavelength of the major band of fluorescence emitted by algae at room temperature (see section 1.5.2) Figure 48 shows the effect of increasing CuSO_4 concentration on the transmission of 695nm light through 0.01M MOPS buffer under similar experimental conditions to those under which fluorescence experiments were carried out. Since increasing copper concentrations above circa $2.0 \times 10^{-4}\text{M}$ decreased the transmission of the 695nm light it was decided that optical measurements made at such high concentrations would be invalid so it was necessary to limit the concentrations of copper added to below this upper limit.

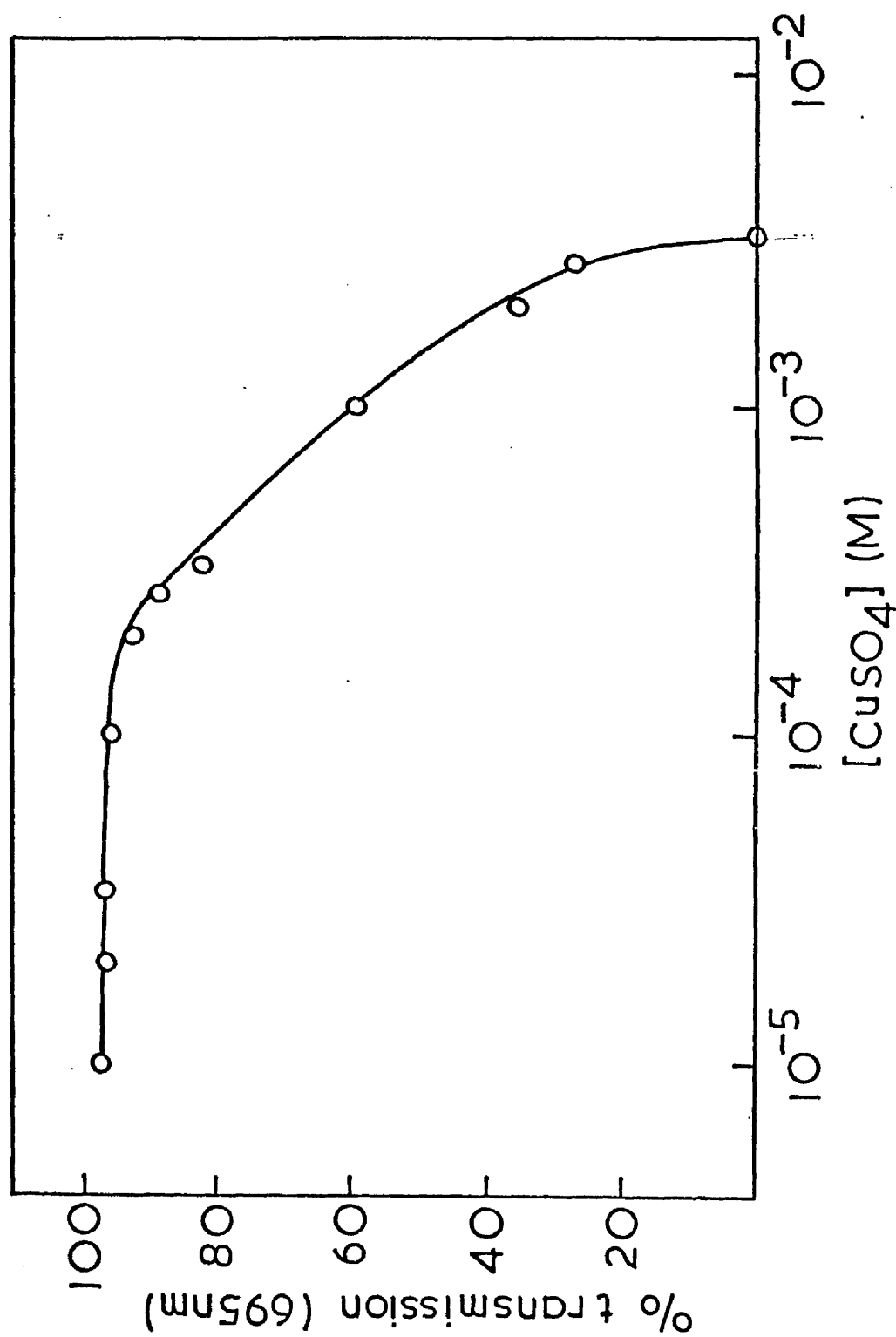


Figure 48. The effect of increasing concentrations of CuSO_4 on the transmission of 695 nm light through a 0.01M solution of MOPS buffer in a 1 cm pathlength cuvette.

In order maximally to inhibit oxygen evolution under such conditions it was necessary to decrease the chlorophyll concentration of the algal suspension according to the data of Table 4. Decreasing the algal concentration however had the disadvantage of decreasing the signal-to-noise ratio of the absorption change measurements (section 4.1.1.6) and so a compromise had to be reached whereby the chlorophyll content was decreased only until oxygen evolution was inhibited in the 10^{-4}M range and no further. The chosen chlorophyll concentration which suited this compromise was $5\text{ }\mu\text{g ml}^{-1}$.

Thus for all the heavy metal studies a chlorophyll concentration of $5\text{ }\mu\text{g ml}^{-1}$ and 0.01M MOPS buffer were employed and the pH adjusted to 6.8; slightly towards the acid side of the pKa. It was hoped that this would allow more of the buffer to become protonated and consequently less likely to bind heavy metals (Raaflaub, 1956).

Figure 49 shows the effect of increasing Cu^{2+} concentration on oxygen evolution by Chlorella under the above conditions; 50% inhibition occurs at circa $3.5 \times 10^{-5}\text{M}$ CuSO_4 and maximal inhibition around $1.5 \times 10^{-4}\text{M}$ CuSO_4 . These results show that copper at these concentrations inhibits photosynthesis in a relatively fast time as distinct from the more long term inhibition effects reported, such as the inhibition of growth, chlorophyll formation, and autospore liberation (Steeman-Nielsen et al. 1969; Sandmann and Böger, 1980a). This copper inhibition affects photosynthetic oxygen evolution and hence electron transport (section 1.5.1) almost immediately and thus its site of action must lie within the chloroplast itself and is therefore distinct from the inhibition of cell division caused by the binding of copper to the cytoplasmic membrane of the algae (Steeman-Nielsen et al. 1969)

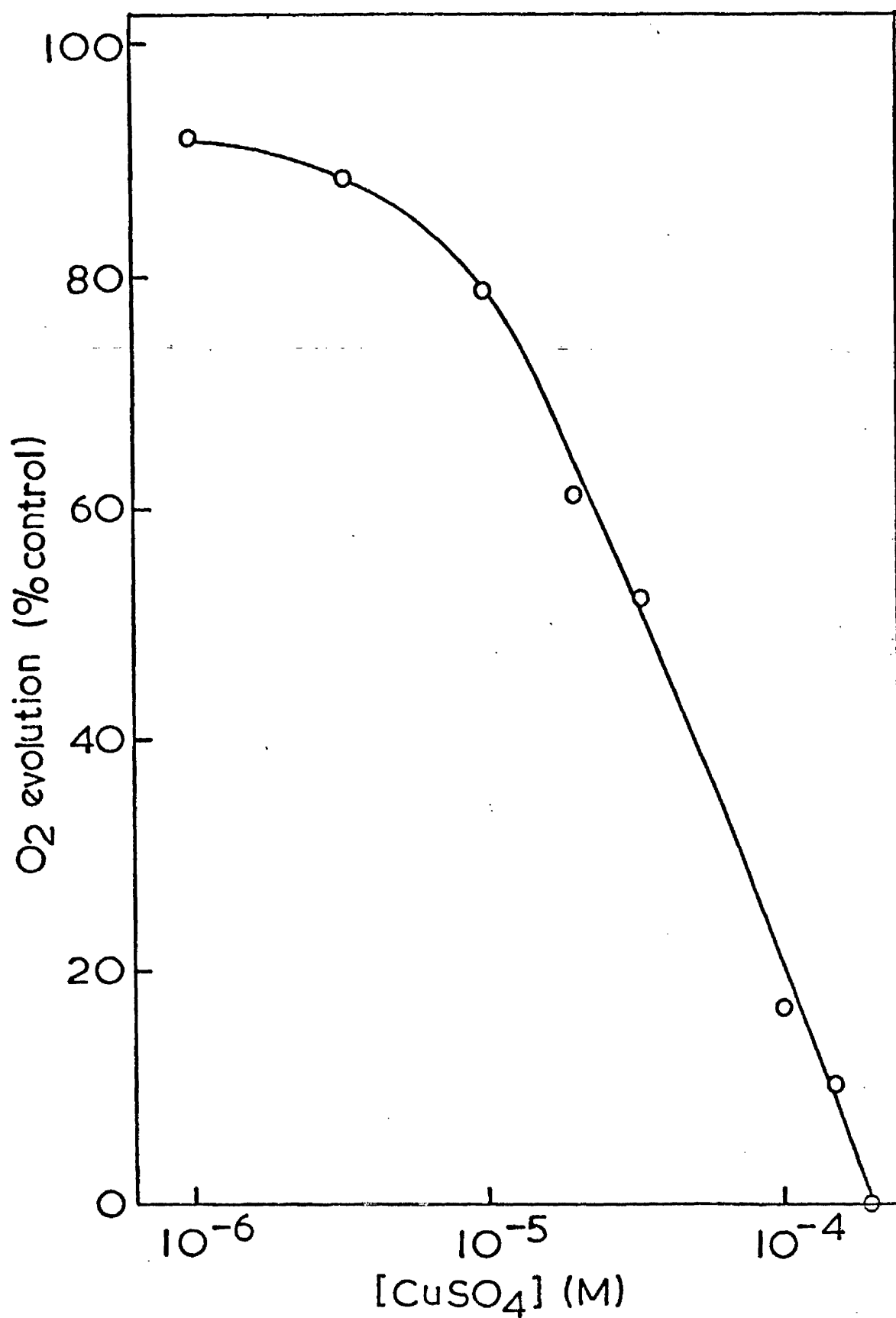


Figure 49. The effect of increasing concentrations of CuSO_4 on the steady state evolution of oxygen in *Chlorella*.

The control rate was $90 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

4.1.1.2 The Effect of Light Versus Dark Incubation of CuSO₄

Steeman-Nielsen et al. (1969) reported that the inhibitory effect of copper on photosynthesis is dependent on whether the algae are pre-incubated in the dark or light. It was therefore decided to investigate the effect of copper concentration under the two different light regimes. The algal cells were preincubated for five minutes with copper in the light or dark and then the cells were illuminated for five minutes to allow a steady rate of oxygen evolution to develop. The results are shown in Table 5. The data indicate that inhibition by the various copper concentrations is more marked when the copper incubation is carried out in the dark rather than in the light. This observation is in contrast with most of the literature (section 1.6) in which it is reported that less copper inhibition occurred in the dark than light. This was taken as evidence that copper binds to inhibitory sites in the light and non-inhibitory ones in the dark (Cedeno-Maldonado et al. 1972). The data shown here tends to suggest the opposite; that copper either binds to the inhibitory sites more strongly in the dark or that the sites of inhibition are altered by light treatment preventing the copper from binding to them. The data shows clearly that copper does inhibit in the dark.

4.1.1.3 The Effect of Copper on Chlorophyll Fluorescence in the Presence of DCMU

A number of reports suggest that copper preferentially inhibits the light reactions of PSII (Cedeno-Maldonado et al. 1972; Haberman, 1969). Furthermore, it has been reported that copper incubation is correlated with chlorophyll destruction (Sandmann and Böger, 1980a). Thus copper might be expected to interfere with the photochemistry of PSII. Fluorescence induction studies provide a method of investigating PSII photochemistry (see section 1.5.2) and thus it was decided to investigate the effect of varying copper concentrations on the fluorescence induction curve in the presence of 10 μ M DCMU.

Table 5

Effect of 5 Minutes Light versus 5 Minutes Dark Incubation
of CuSO₄ on Steady State Oxygen Evolution by Chlorella

<u>CuSO₄ Addition (M)</u>	<u>O₂ Evolution (% Control)</u>	
	<u>5 Mins Dark Incubation</u>	<u>5 Mins Light Incubation</u>
10 ⁻⁵	90.0	90.5
2 x 10 ⁻⁵	64.3	79.3
3.3 x 10 ⁻⁵	49.3	73.2
10 ⁻⁴	4.0	15.5

The control rates were 252 $\mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$. and 263 $\mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$. for the light and dark incubations respectively.

Figure 50 shows the effect of copper on the three fluorescence parameters (i) F_0 , which represents changes in the rate constants of deexcitation either in pigments not associated with PSII or in the PSII antennae pigments before the exciton has reached the reaction centre (ii) F_m which represents changes in fluorescence quenching caused by the closing of traps at the reaction centre (iii) $(F_m - F_0)/F_m$ which represents changes in the overall efficiency of energy trapping by the reaction centre either as changes in the quantum yield of photochemistry or as changes in the rate constant for radiationless decay at the reaction centre as discussed in section 1.5.2 (Butler, 1978). The data shows that all three parameters decrease over a similar concentration range as oxygen evolution is inhibited. The F_m parameter would seem to be inhibited more strongly than the other two parameters.

It should be noted that the copper-induced quenching of fluorescence is not thought to be due to the absorption of the red fluorescence by the blue copper sulphate solution since the absorption of 695nm light is not affected by the addition of copper sulphate of concentrations of $\leq 2.0 \times 10^{-4}$ (Transmission 695nm = 96.0% at 10^{-4} CuSO₄ added to 3ml buffer sample) (see Figure 48).

4.1.1.4 The Effect of Copper on Chlorophyll Fluorescence in the Absence of DCMU

To monitor the rate of electron transport through PSII by the Hill reaction using artificial electron acceptors such as ferricyanide or DCPIP which is the normal practice in isolated chloroplasts, is more difficult in intact algal cells. The problem of permeability of the cell towards these electron acceptors is encountered again and so an in vivo method is necessary. Although still not fully understood the fluorescence induction curve in the absence of DCMU is generally interpreted to reflect the redox state of Q, the primary electron acceptor of PSII, under the influence of electron transport (see section 1.5.2.4).

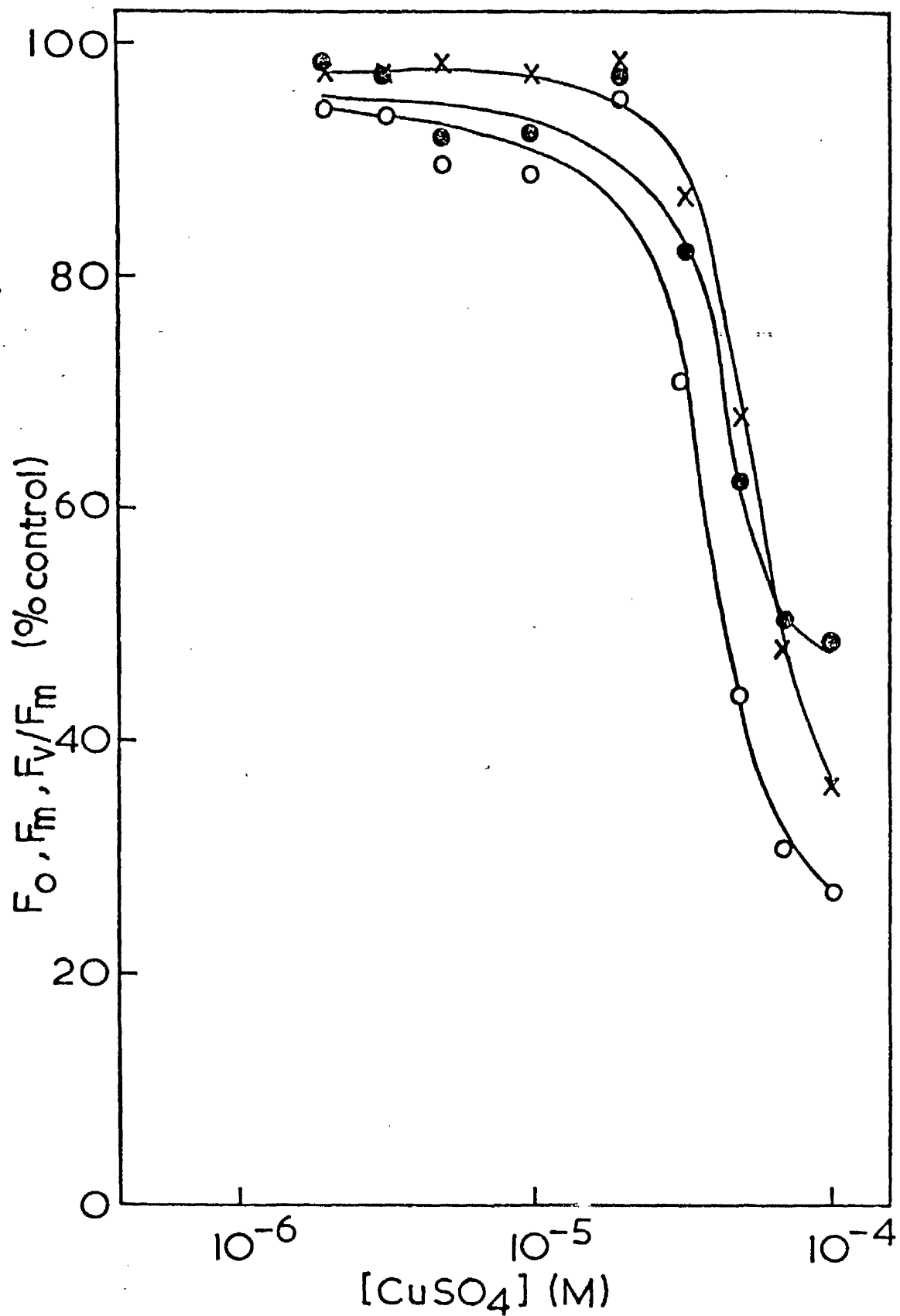


Figure 50. The effect of increasing concentrations of CuSO_4 on the chlorophyll fluorescence induction parameters F_0 (●), F_m (○) and F_v/F_m (x) in *Chlorella* after 5 minutes dark adaption in the presence of 10 μM DCMU.

Therefore, as there is no electron block after Q (i.e. DCMU) then the redox state of Q will be dependent on the rates of electron input from the water splitting side of PSII and also the rates of secondary electron transfer from Q to the secondary acceptors in the intersystem electron transport chain. The fluorescence will also be sensitive to changes in the photophysical events of light harvesting and exciton transfer as discussed in the previous section. Slower changes reflect different types of mechanism such as the induction of the high energy state and other slow processes involved in the induction of overall photosynthesis and are not considered here.

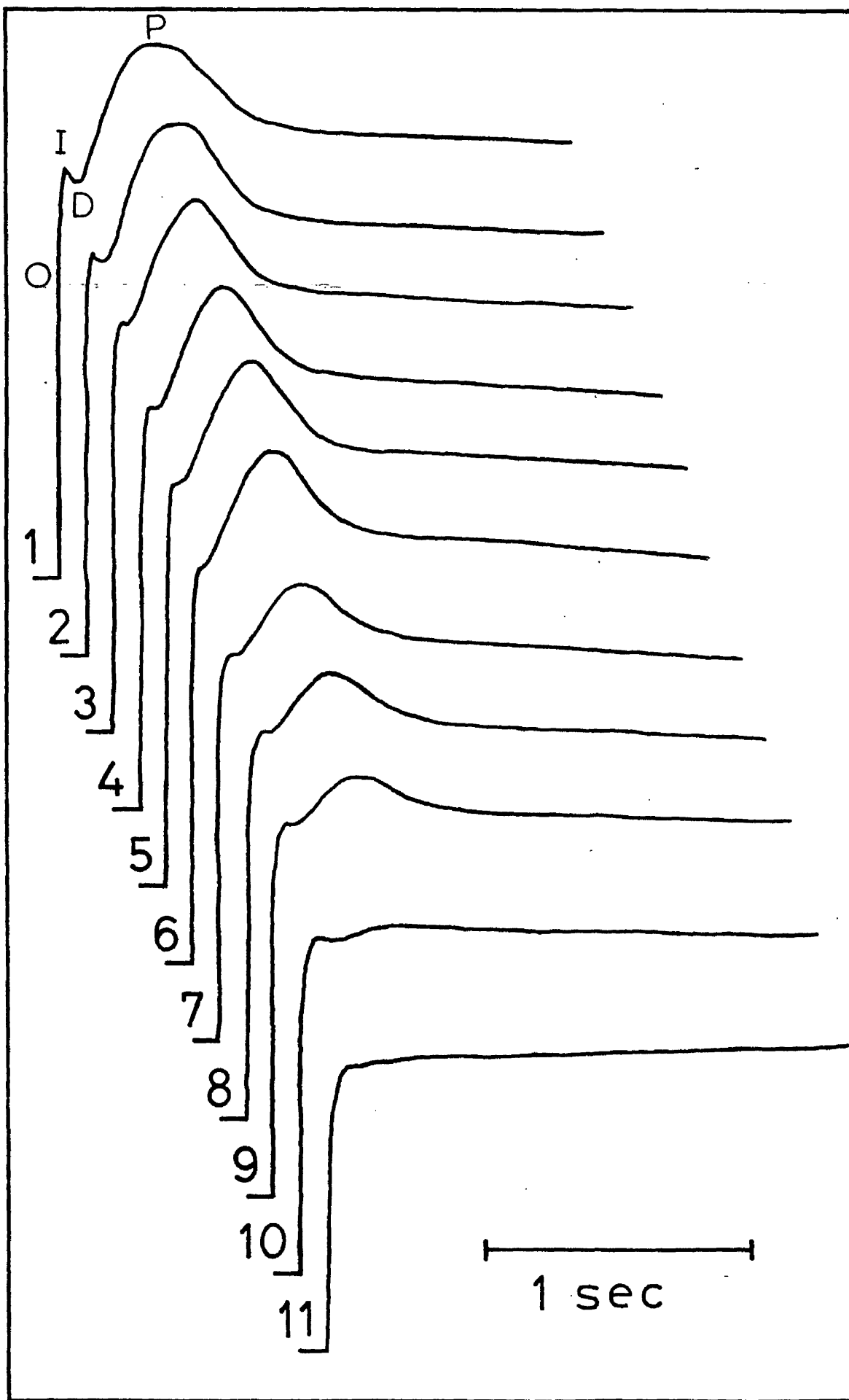
Figure 51 shows the fluorescence induction of Chlorella which have been dark adapted for 5 minutes, after incubation with increasing concentrations of CuSO_4 . Three main effects can be observed when the copper concentration is increased, firstly at low copper concentrations which only partially inhibit oxygen evolution ($\geq 2.0 \times 10^{-5}\text{M}$) the I - D dip becomes less prominent, secondly at concentrations which largely inhibit oxygen evolution (greater than $8.3 \times 10^{-5}\text{M}$) the maximum peak, D-P-S, is increasingly quenched, and thirdly at strongly inhibitory concentrations (greater than $1.2 \times 10^{-4}\text{M}$) the initial O and I levels are increasingly quenched.

4.1.1.5 The Effect of Copper on Oxygen Evolution induced by Flash Trains

As described in section 1.5.1.1 a further method for studying the light reactions of PSII is provided by flash-induced oxygen evolution. The normal procedure for studying the effects of inhibitors etc on the flash-induced oxygen evolution pattern is to introduce the compound into the gravity fed buffer solution from where it can diffuse across the membrane into the reaction cell and thus exert its effect on the photosynthetic material. This avoids fresh algal (or chloroplast) samples having to be used each time a measurement is taken and also the flash patterns before and after the addition may be compared directly.

Figure 51. The effect of increasing concentrations of CuSO_4 on the chlorophyll fluorescence induction in the absence of DCMU (Kautsky phenomena) in Chlorella after 5 minutes dark adaption. Final concentrations of CuSO_4 were; 1, no addition; 2, $3.3 \times 10^{-6}\text{M}$; 3, $2.0 \times 10^{-5}\text{M}$; 4, $2.7 \times 10^{-5}\text{M}$; 5, $3.3 \times 10^{-5}\text{M}$; 6, $6.7 \times 10^{-5}\text{M}$; 7, $8.3 \times 10^{-5}\text{M}$; 8, $1.0 \times 10^{-4}\text{M}$; 9, $1.2 \times 10^{-4}\text{M}$; 10, $1.3 \times 10^{-4}\text{M}$; 11, $2.0 \times 10^{-4}\text{M}$.

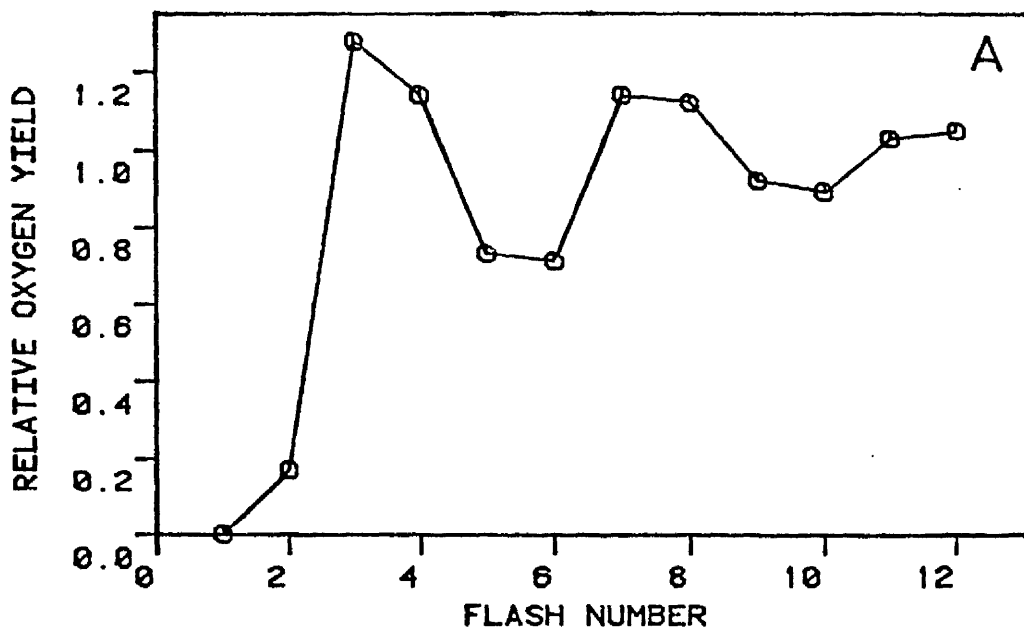
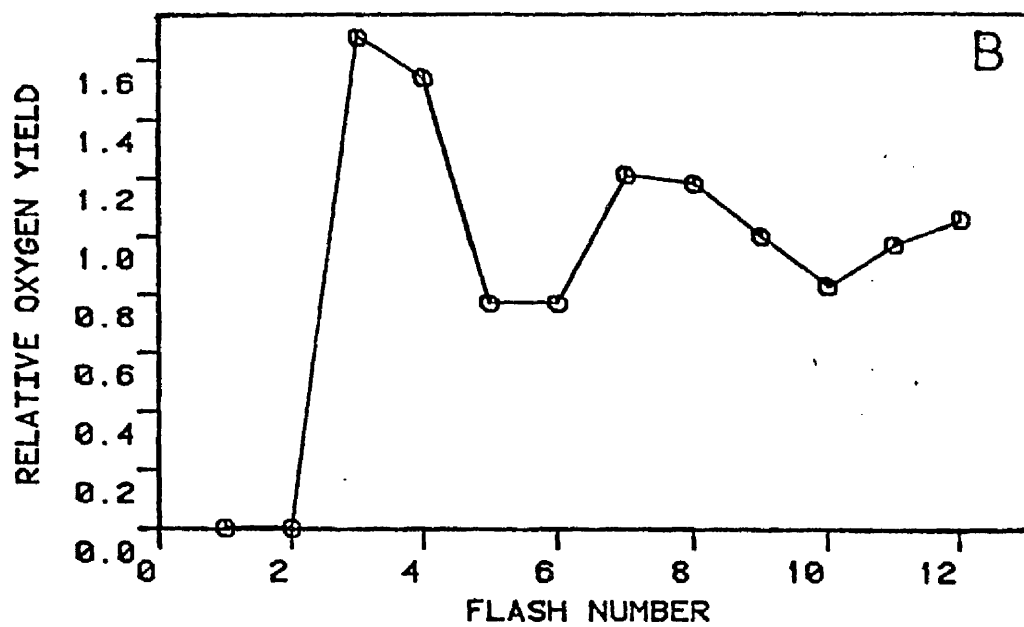
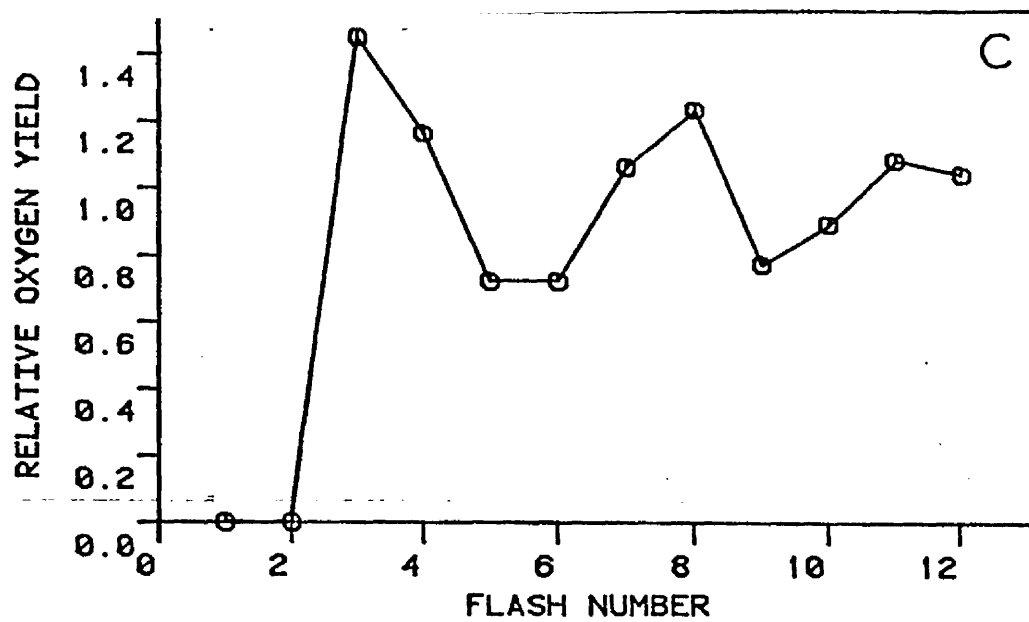
relative fluorescence



The extents of the flash-induced oxygen transients cannot be compared between different algal samples, since the electrode has to be taken apart and a new sample placed on top of the cathode, only the overall pattern of the oxygen transients. It was thus hoped to be able to introduce copper into the algal sample via the gravity-fed buffer. However, it was found that the free CuSO_4 solution underwent electrolysis almost immediately and copper became deposited on the platinum cathode preventing the electrode from functioning. Therefore a different technique had to be employed; the algal samples were first incubated for five minutes in appropriate copper concentrations and then centrifuged, washed, centrifuged and resuspended in MOPS buffer. The cells were then immediately placed on the electrode and the resulting oxygen evolution pattern measured. In order to check that the copper inhibition still occurred after washing, some of the resuspended algae were placed in a steady-state oxygen evolution cell and the resulting inhibition measured as discussed in section 4.1.1.1. In this way the inhibition was still found to occur after the cells had been washed. Therefore the flash-induced oxygen transients shown in Figure 52 can only be compared with respect to each other qualitatively by the amounts involved per flash and not by the quantitative amounts of oxygen evolved.

The data in Figure 52 shows that no marked variation in the relative amounts of oxygen evolved per flash was observed when the algae were incubated in copper concentrations up to $1.25 \times 10^{-4}\text{M}$. Very high copper concentrations inhibited flash-induced oxygen evolution altogether. Whether this is a real effect or whether an artifact induced by copper bound to or within the algae being released into the resuspending solution during the experiment and reacting with the cathode remains unclear. Slight traces of copper were found to be deposited after algae incubated in high copper concentrations had been used suggesting it probably was an artifact.

Figure 52. The effect of increasing concentrations of CuSO_4 on the yields of oxygen produced by a series of short saturating flashes, spaced 500 ms apart, in Chlorella after 5 minutes dark adaption. Relative oxygen yields represented as the yield per flash (Y_n) divided by the steady state yield (Y_{ss}) derived from the mean value of the 13th to 18th flash yields. A, no additions; B, with the addition of $7.5 \times 10^{-5} \text{ M CuSO}_4$; C, with the addition of $1.25 \times 10^{-4} \text{ M CuSO}_4$.



4.1.1.6 The Effect of Copper on the Absorption Change at 515nm

The absorption change at 515nm represents an electrochromic response to the field generated across the thylakoid membrane as already discussed (see section 1.5.3.1). Therefore in order to discover whether copper had any effect on the generation and decay of the electric field across the membrane the effect of copper concentration was investigated. Algae were incubated in the dark for five minutes with varying copper sulphate concentrations before the flash-induced absorption change was measured. Figure 53 shows the effect of increasing CuSO_4 concentration on the fast and slow phases of absorption increase and the half time of the overall decay rate. Two effects are observed. Firstly, at concentrations which inhibit oxygen evolution (circa 10^{-4}M), the extent of the slow phase is increased and the rate of decay markedly decreased. Secondly at similar concentrations, and it should be noted not just at higher concentrations, the extent of the fast phase is decreased with increasing copper. The first of these two effects is similar to that observed with low uncoupler concentrations (see Chapter 3). This suggests that copper also might act as an uncoupler, however, in the case of the CCCP studies higher concentrations induced uncoupling effects of increasing the rate of decay due to inducing greater permeability of the thylakoid allowing the high energy intermediate to dissipate more rapidly. It was not possible, however, to use higher copper concentrations since, as discussed in section 4.1.1.1, this would have induced artifacts from precipitates forming between copper and the buffer. It is assumed that such findings might occur as high copper concentrations have been shown to increase the rate of decay of the absorption change in chloroplasts (Hipkins, unpublished results).

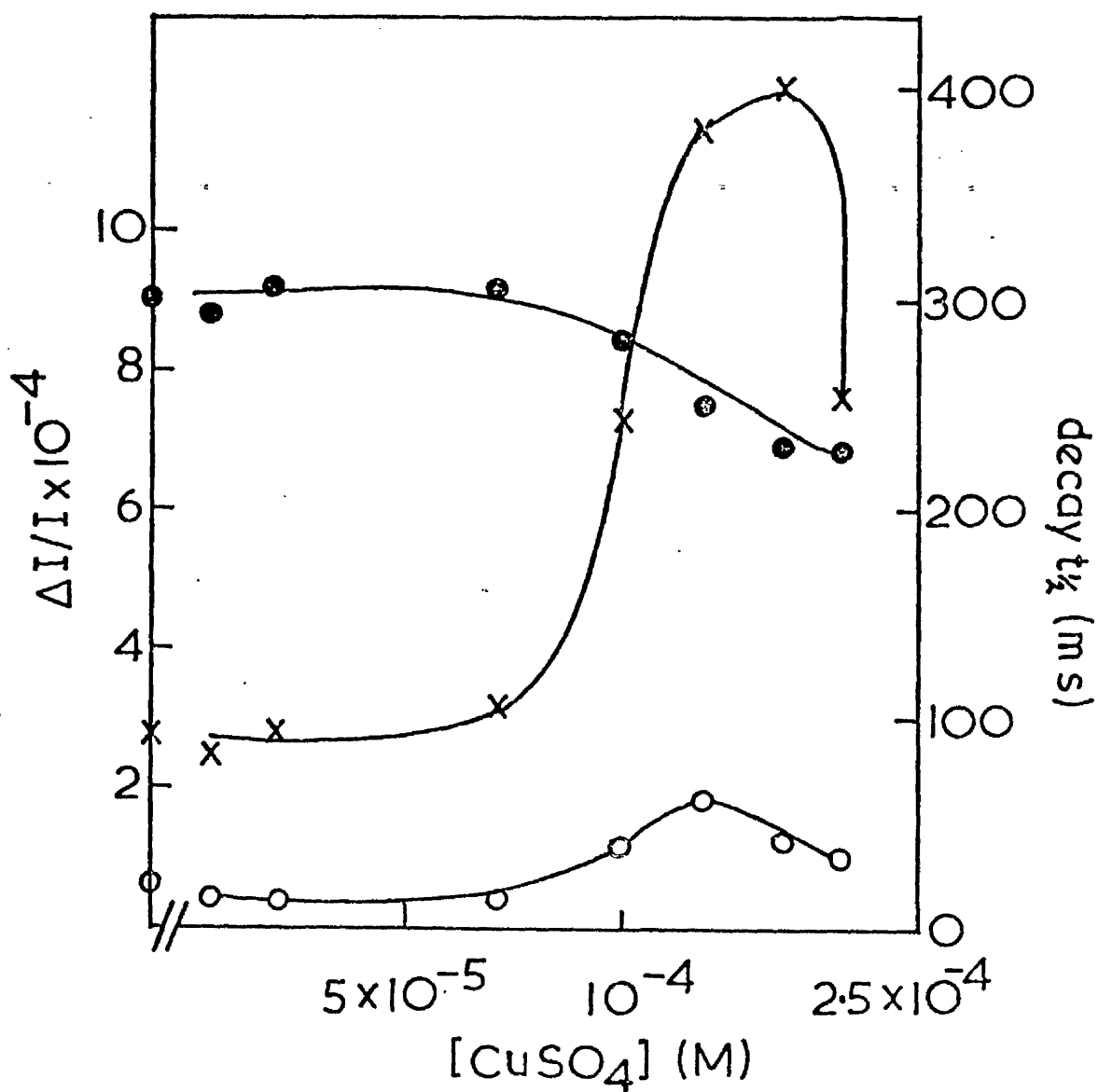


Figure 53. The effect of increasing concentrations of CuSO_4 on the fast phase (o), slow phase (•) and decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella* after 5 minutes dark adaption. Each point represents the accumulated average of 128 sweeps at a flash frequency of 0.5 Hz.

As in the studies on the effect of uncouplers on the 515 absorption change (Chapter 3) deconvolutions of (i) a control trace and (ii) a typical trace showing the increased extent of the slow phase and decreased rate of decay, brought about by the addition of $1.3 \times 10^{-4} \text{M}$ CuSO_4 , were carried out. The data are shown in Table 6 and indicate as shown for the uncouplers that the rate of decay is markedly decreased and the slow phase is stimulated.

Since copper has been suggested to quench the excitons in the collector pigments (see section 4.1.1.3) then this might be expected to decrease the amount of charge separated at the reaction centres. To investigate this further the effect of blocking the photochemical reactions of PSII with $10 \mu\text{M}$ DCMU and $100 \mu\text{M}$ NH_2OH (section 3.1.5) was studied on the extent of the fast phase. The results of the effect of increasing copper concentrations on the fast phase of absorption increase in the presence and absence of DCMU and NH_2OH are depicted in Table 7. The data show that as in Figure 53 the effect of increasing copper concentration in the absence of DCMU and NH_2OH is to inhibit the overall extent of the fast phase of absorption increase. However when PSII is blocked then the extent of the fast phase is more markedly decreased, and almost totally inhibited at high concentrations. Therefore, these data suggest that when PSII is blocked, copper inhibits the charge separation at PSI also.

4.1.1.7 The Effect of Magnesium Sulphate on Oxygen Evolution

In order to ensure that it was the copper II ions which were the toxic elements in photosynthesis of Chlorella and not the sulphate anions a control curve was determined by incubating Chlorella with varying concentrations of MgSO_4 over the same concentration range as was used for CuSO_4 . The data (Figure 54) show that no effect on oxygen evolution was observed even with concentrations as high as $2.0 \times 10^{-2} \text{M}$ MgSO_4 , under the same experimental conditions as those employed for the CuSO_4 incubations.

Table 6

Deconvolution of Typical Experimental Traces of the Flash-Induced Electrochromic Absorption Change under (a) Control Conditions and (b) in the Presence of 1.3×10^{-4} M CuSO_4 , According to a Linear Combination of Two Exponentials: $\Delta A(t) = \{ A + B(1 - \exp(-kt)) \} \exp(-t/\tau)$.

Treatment	$A(\Delta I/I \times 10^{-5})$	$B(\Delta I/I \times 10^{-5})$	$k(\text{msec})$	$\tau(\text{msec})$
(a) Control	12.0 ± 1.6	2.0 ± 1.5	3.70 ± 5.19	83.30 ± 5.49
(b) + 1.3×10^{-4} M CuSO_4	9.6 ± 0.91	3.89 ± 0.88	7.77 ± 5.03	343.17 ± 63.75
(b)/(a) x 100%	80.0	194.5	210.0	412.0

Table 2

The Effect of Increasing Concentration of CuSO_4 on the Fast Phase
of Absorption Increase at 515nm in the Presence and Absence of $10\mu\text{M}$
DCMU and $100\mu\text{M}$ NH_2OH

CuSO_4 (M)	Extent of Fast Phase of $\Delta 515$			
	No Addition		+ $10\mu\text{M}$ DCMU, $100\mu\text{M}$ NH_2OH	
	$\Delta I/I \times 10^{-5}$	% Control	$\Delta I/I \times 10^{-5}$	% Control
Control	8.57	100	5.68	66.3
+ 1.0×10^{-4}	8.14	95.0	5.57	65.0
+ 1.3×10^{-4}	6.75	78.8	4.12	48.1
+ 1.7×10^{-4}	5.25	61.3	1.02	11.9
+ 2.6×10^{-4}	5.35	62.4	0.86	10.0

The algae were incubated for 5 minutes in the dark with the various CuSO_4 concentrations, after 10 seconds pre-illumination (2 x Schott RG 680 red filters, giving an intensity of 60 Wm^{-2}) in the absence or presence of $10\mu\text{M}$ DCMU and $100\mu\text{M}$ NH_2OH . Each trace represented the accumulated average of 128 sweeps at a flash frequency of 0.5 Hz.

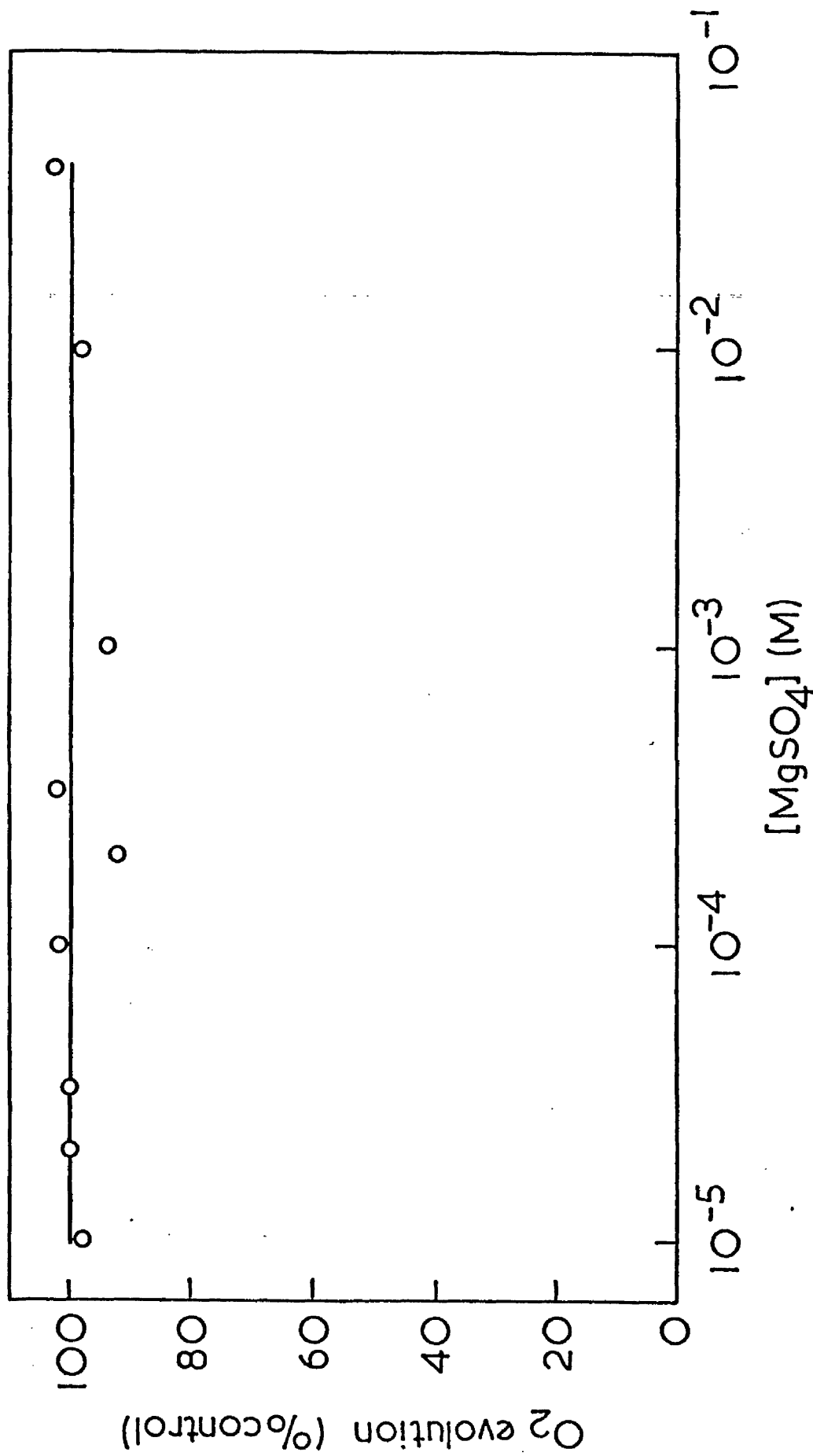


Figure 54. The effect of increasing concentrations of MgSO_4 on the steady state evolution of oxygen in Chlorella.

The control rate was $170 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

Therefore it is suggested that it is the copper II ions which are toxic and not the sulphate anions.

4.1.2 Discussion

The various pieces of data shown for the effects of copper on the light reactions indicate several possible sites for copper action. These are now discussed. Firstly, at increasing concentrations of copper from circa $10^{-6}M$ the steady state flow of electrons from the water splitting complex to the Calvin cycle is increasingly inhibited until total inhibition occurs at concentrations greater than $10^{-4}M$, at algal chlorophyll concentrations of $5\mu g\ ml^{-1}$. This inhibition occurs both in the dark or in the light and is dependent on the copper to chlorophyll ratio as well as the copper chelating capacity of the suspending medium of algae. Furthermore, the inhibition occurs rapidly, within 5 minutes in the experiments carried out here, and is therefore distinct from the slow inhibition of growth and cell division reported by Steeman-Nielsen et al. (1969) and Steeman-Nielsen and Wium-Andersen (1970) although inhibition of photosynthesis may itself lead to longer term inhibition by preventing the production of photosynthetic assimilates which are needed for cellular growth.

The inhibition of photosynthesis by copper was investigated on PSII activity since this site has been reported to be particularly sensitive to heavy metals in isolated chloroplasts (see Cedeno-Maldonado et al. 1972; Tripathy and Mohanty, 1980; Miles et al. 1972). The data of the effect of copper on fluorescence, both in the presence and absence of DCMU, and on the flash-induced oxygen evolution patterns suggest that copper inhibits PSII either by acting as an external quencher of exciton energy or possibly by blocking electron flow on the donor side of the reaction centre. The quenching of the F_0 , F_m and F_v/F_m levels of fluorescence suggests that copper provides an extra pathway for the deexcitation of excited singlet chlorophyll in the light harvesting pigments.

This type of quenching has been reported by Etienne et al. (1974) for the compound di-nitrobenzene and they suggest that this compound has a quenching effect both on the reaction centre chlorophyll and on the light harvesting chlorophyll of the PSII photosynthetic units. Kitajima and Butler (1975) also reported a quenching of F_0 , F_m , and F_v/F_m levels by DBMIB at low temperatures. They suggest that two types of quenching can be recognised (i) increasing non-radiative decay processes in the bulk chlorophyll by creating quenching centres which compete with the reaction centres for the excitation energy inducing quenching of F_m , F_0 and F_v and (ii) increasing non-radiative decay at the reaction centre which induces quenching of F_v and F_m but not F_0 . The copper-induced quenching seen in Figure 50 is clearly of the former type as both F_0 and F_m are quenched.

This quenching of the fluorescence parameters F_0 , F_m and F_v/F_m may be analysed in terms of the rate constants for depopulation of the first excited singlet state of chlorophyll as shown for DBMIB by Kitajima and Butler (1975) and Butler and Kitajima (1975) by assuming that Cu^{2+} acts as a typical external quencher of in vivo fluorescence. Both models of energy transfer between the light harvesting pigments and the reaction centre described in section 1.5.2.2 may be equally applied as follows. Firstly, the more simple theory may be applied where the ratio F_v/F_m is taken to be a reliable index of the maximum yield of photochemistry of PSII, ϕ_{pmax} , and thus reaction centres not capable of photochemistry are assumed not to be able to accept excitons from the light harvesting pigments. It was shown in section 1.5.2.2 that the quantum yield of fluorescence at the F_0 and F_m levels and the quantum yield of photochemistry may be represented by the rate constants of de-excitation of the excited chlorophyll molecules as follows:

$$\phi_{F_0} = \frac{k_f}{k_f + k_h + k_t + k_p}$$

$$\phi_{F_m} = \frac{k_f}{k_f + k_h + k_t}$$

$$\frac{\phi_{F_m} - \phi_{F_0}}{\phi_{F_m}} = \frac{F_V}{F_m} = \frac{k_p}{k_f + k_h + k_t + k_p} = \phi_{pmax}$$

The quenching of fluorescence by Cu^{2+} may be represented by an additional rate constant for de-excitation k_q . This extra quenching rate constant could equally well be an increase in the rate constants k_h or k_t since the experiments carried out here cannot differentiate between these different possible fates of exciton energy. By placing this extra term in the above equations we obtain expressions for ϕ_{F_0} , ϕ_{F_m} and ϕ_{pmax} in the presence of the quencher Cu^{2+} :

$$\phi_{F_0} = \frac{k_f}{k_q + k_f + k_h + k_t + k_p}$$

$$\phi_{F_m} = \frac{k_f}{k_q + k_f + k_h + k_t}$$

$$\frac{\phi_{F_m} - \phi_{F_0}}{\phi_{F_m}} = \frac{F_V}{F_m} = \frac{k_p}{k_q + k_f + k_h + k_t + k_p}$$

Thus it can be seen that the addition of the k_q term by an external quencher, i.e. Cu^{2+} , to each of these three equations would induce a decrease in the three values of ϕ_{F_0} , ϕ_{F_m} and ϕ_{pmax} and the three curves in Figure 50 show that this is the case.

Assuming that the rate constant for photochemistry is much greater than the other rate constants (Butler and Kitajima, 1975) then the yield of the three parameters might however be expected to be decreased by varying amounts.

This is because the presence of the relatively large k_p terms in the expressions for ϕ_{F_0} and ϕ_{pmax} would act in a buffering manner reducing the effect of the additional relatively small k_q term whereas the expression for ϕ_{F_m} contains no large buffering term and the addition of the k_q term would thus have a greater effect on ϕ_{F_m} . This assumes that k_q is of a relatively similar size to the non photochemical rate constants.

The data of Figure 50 also concur to some degree with this as the F_m term shows a greater sensitivity towards increasing Cu^{2+} concentration with respect to the ϕ_{F_0} and ϕ_{Pmax} values.

As mentioned in section 1.5.2.2 this simple model has recently been questioned by a number of groups due to the finding that values of the quantum yield of photochemistry ϕ_{Pmax} , derived from the expression $\phi_{Pmax} = (\phi_{F_m} - \phi_{F_0})/\phi_{F_m}$ from fluorescence induction measurements are rather low compared to values of the quantum yield of photochemistry as measured by electron transport (Haehnel et al. 1982). Furthermore a number of treatments such as ferricyanide addition, washing with strong concentration of TRIS or irradiation with ultraviolet light induce changes in the ϕ_{Pmax} values derived from the F_v/F_m expression which do not correlate with those determined from electron transport (Butler and Kitajima, 1975). These discrepancies have been accounted for by Butler and Kitajima (1975), Van Grondell and Duysens (1980) and Haehnel et al. (1982) by assuming a new radiationless deactivation pathway in closed reaction centres, k_d . Therefore even closed reaction centres may accept excitons and therefore fewer excitons will be available to denonulate via the other pathways with rate constants k_f , k_h , and k_t . This would lead to a marked decrease in the final level of maximum fluorescence F_m without causing much change in the F_0 level which contains the large buffering photochemical rate constant, k_p .

Therefore the equations for ϕ_{F_0} , ϕ_{F_m} and $(F_m - F_0)/F_m$ must be treated slightly differently under this type of model when the effect of an external quencher, as Cu^{2+} is suggested to act here, is investigated. By replacing the rate constant for exciton trapping at the reaction centre k_p with the rate constant k_T' where $k_T' = k_T [A + (1 - A)\phi_d]$ and A is the fraction of open trans and ϕ_d is the quantum yield for radiationless decay at the reaction centre, expressions for ϕ_{F_0} , ϕ_{F_m} and F_v/F_m may be obtained as follows (Butler and Kitajima, 1975) (see appendix 5).

$$\phi_{F_0} = \frac{k_f}{k_f + k_h + k_t + k_T [1]}$$

$$\phi_{F_m} = \frac{k_f}{k_f + k_h + k_t + k_T [\phi_d]}$$

$$\frac{\phi_{F_m} - \phi_{F_0}}{\phi_{F_m}} = \frac{F_v}{F_m} = \frac{k_T [1] - k_T [\phi_d]}{k_f + k_h + k_t + k_T [1]} = \frac{k_T (1 - \phi_d)}{k_f + k_h + k_t + k_T [1]} = \phi_T (1 - \phi_d)$$

Assuming that the rate constant for photochemistry is much greater than the rate constant for radiationless decay at the reaction centre then ϕ_T is still a good estimate of ϕ_{pmax} , the quantum yield of photochemistry. If the radiationless decay increases however then the values obtained for F_v/F_m and F_m will decrease, increasing the difference between ϕ_T and ϕ_{pmax} .

If Cu^{2+} acts as an external quencher, providing an extra pathway for exciton de-excitation, then as in the previous model, an extra rate constant for this quenching k_q may be placed in the expressions for ϕ_{F_0} , ϕ_{F_m} and F_v/F_m as follows

$$\phi_{F_0} = \frac{k_f}{k_q + k_f + k_h + k_t + k_T [1]}$$

$$\phi_{F_m} = \frac{k_f}{k_q + k_f + k_h + k_t + k_T [\phi_d]}$$

$$\frac{\phi_{F_m} - \phi_{F_0}}{\phi_{F_m}} = \frac{F_v}{F_m} = \frac{k_T (1 - \phi_d)}{k_q + k_f + k_h + k_t + k_T [1]}$$

Therefore assuming Cu^{2+} does not alter the rate of radiationless decay at the reaction centre, the same result would be expected from adding the extra k_q term, as in the previous model, i.e. a decrease in the three parameters ϕ_{F_0} , ϕ_{F_m} and F_v/F_m with the ϕ_{F_m} parameter being more sensitive to copper induced quenching as it does not contain a relatively large photochemical rate constant. That the ϕ_{F_0} values decrease with copper addition further supports the fact that it is not having an inhibitory effect on ϕ_d as this term is assumed to be negligible or zero in the expression for ϕ_{F_0} .

Both these analyses assume that energy transfer within the photosynthetic apparatus is of the "lake" as opposed to "puddle" method. The puddle model assumes that the apparatus consists of independent photosynthetic units with one reaction centre per unit and no energy transfer between units whereas the lake model assumes unrestricted energy transfer between photosynthetic units (Govindjee and Govindjee, 1975). The lake model has been adopted here for its more simple analysis but the addition of an extra de-excitation term may be applied to the puddle model with the same effect on the above parameters F_0 , F_m and F_v/F_m (Butler and Kitajima, 1975).

Therefore the effect of Cu^{2+} on fluorescence in the presence of DCMU is accounted for by it acting as an external quencher i.e. an extra pathway for energy de-excitation in the collector pigments. This reduces the singlet excitation energy, absorbed by the light harvesting pigments, that is available for photochemistry at the reaction centre. The quenching may be due to copper itself or alternatively copper may be acting by producing ultra-structural changes in the thylakoid membranes that allow penetration of excitation quenchers to the pigment bed itself. If the F_0 fluorescence is assumed to emanate from pigments other than PSII then copper may be seen to add an extra quenching centre to these pigments also. If some of this F_0 fluorescence originates in pigments associated with PSI, as proposed by Clayton (1969) and Telfer et al. (1978) then copper might be expected to inhibit the photochemical reactions of PSI as well as PSII. Evidence supporting this hypothesis is presented in the data showing the effect of copper on the absorption change at 515nm.

Another possible mechanism for quenching of fluorescence in the presence of DCMU is that copper prevents the reduction of Q by blocking electron donation to the reaction centre from the water-splitting complex.

This would result in a decrease in the variable fluorescence and the F_m level since the oxidised reaction centre chlorophyll or primary electron donor would not be rereduced by electrons from the water-splitting complex. Therefore the separated charges might be expected to recombine with the production of a triplet or ground state chlorophyll. Although this energy loss would quench the variable fluorescence it would not be expected to quench the initial non-variable fluorescence level F_0 . Since copper does decrease the F_0 level the probability of copper inhibiting the donor side of PSII is therefore considered unlikely to be the cause of the quenching of the fluorescence in the presence of DCMU. Tripathy and Mohanty (1980) found that zinc inhibited the donor side of PSII and consequently quenched the variable fluorescence; they also reported that the F_0 level was not affected. This concurs with the above conclusion that copper quenches by a means other than by blocking electron donation to the PSII reaction centre.

A similar mechanism, although not the only one possible, is suggested by the effects of copper on the fluorescence induction in the absence of DCMU. An overall quenching of the induction was observed with the I-D-P transients being more sensitive at low concentrations in a similar manner to the F_m levels. Strongly inhibiting copper concentrations (greater than $1.1 \times 10^{-4} M$) decreased the O and I levels also. Etienne and Laverne (1972) showed that the external quencher 'dinitrobenzene' induced the same type of inhibition as shown here for copper lending further support to the copper inhibitory mechanism suggested above.

However other possible explanations of this copper-induced inhibition in the absence of DCMU must also be considered. Assuming that copper inhibits electron transport through PSII and if copper decreased the pressure of electrons coming from the donor side of PSII, Q would not become as fully reduced before electrons were transferred to the secondary acceptors in the intersystem pool.

This would lead to the P transient decreasing as electron donation to Q became rate limiting rather than the donation from Q to PSI. Higher copper concentrations would altogether prevent electrons being donated to PSII by the water splitting complex and so Q would hardly become reduced at all and thus the 0 - 1 rise would be inhibited. Bohner et al. (1980) have shown that increasing concentrations of copper decrease the maximum fluorescence level, P, in Scenedesmus acutus with only a weak inhibition of the other transient levels, and suggest that this is caused by copper inhibiting the water-splitting activity so that electron pressure from the PSII donor side becomes insufficient to reach the high transient level of reduced Q which results in the P level of fluorescence. Since a similar effect is caused by copper, as shown in Figure 51 the possibility of copper acting as above cannot be ignored. The 1 - D dip seems to show a higher sensitivity to copper than the other levels and maybe tentatively explained in the same way as above. Since the 1 - D dip is thought to be due to Q being reoxidised by the intersystem pool the copper-induced reduced electron pressure from the donor side of PSII would result in Q not being as fully reduced before electrons were transferred to the secondary acceptors and a consequent loss of the 1 - D dip.

Increasing rates of reoxidation of Q would also result in a quenching of the induction curve if copper stimulated rates of electron transfer toward PSI. However since the data on fluorescence with electron flow from Q to the secondary acceptors blocked with DCMU shows a copper-induced quenching this hypothesis is probably ruled out.

If copper does affect the water-splitting mechanism then it might be expected to cause changes in the pattern of flash-induced oxygen yields. However since copper did not seem to affect the actual pattern of the flash oxygen yields there does not seem to be any direct evidence for copper inhibiting the water splitting complex.

Compounds which act as external quenchers of exciton energy such as dinitrobenzene have been shown to reduce the oscillation amplitude but not to deform the flash pattern (Etienne et al. 1974). Since amplitude measurements were not valid for the flash oxygen experiments carried out here the copper data obtained can only suggest that it is compatible with such a hypothesis but not prove that copper exerts its effect on PSII by acting as an external quencher.

The data concerning the effects of copper on the absorption change at 515nm suggested two possible inhibition mechanisms; firstly that copper blocks the primary charge separation at the reaction centres particularly that associated with PSI, and secondly, that copper exerts an uncoupling effect similar to CCCP as already discussed.

Similar explanations for those given for the low uncoupler concentration effects in the previous chapter, may thus be applied here; namely that copper decreases the rate of decay which therefore allows the slow phase to be more easily seen or that copper interferes with the field controlling the redox noise of the components responsible for the electrogenic slow increase.

A possible mechanism by which copper may decrease the rate of decay of the absorption change has been recently reported by Uribe and Stark (1982). They have shown that copper is an extremely effective agent for energy dependent inactivation of phosphorylation. They suggest that copper reacts with the CF₁ coupling factor component probably by causing an oxidation of sulphydryl groups on the coupling factor, which are essential to catalytic function. Since a decrease in the decay rate of the absorption change at 515nm suggests a decrease in the efflux of ions across the membrane, possibly protons during phosphorylation, then the block of the coupling factor and the prevention of proton efflux during phosphorylation might explain the copper-induced decrease in the rate of decay.

A difference between the effects of copper and the phenolic uncouplers is observed though; this is the decrease in the extent of the fast phase at concentrations below and equal to those which stimulate the slow phase and decrease the rate of decay. This suggests an extra copper effect being superimposed on the one described above, namely an inhibition of the charge separation at the reaction centres of PSII or PSI.

Shioi et al. (1977) have shown that copper at concentrations of $2 \times 10^{-6}\%$ inhibits electron transport through PSI as measured by the flow of electrons from DCPIP and ascorbate to NADP. They suggest that copper inhibits PSI at the site of ferredoxin and the NADP reductase enzyme since electron transport with methyl viologen (MV) as the electron acceptor is not affected by copper. Electron flow through PSI to MV requires the primary electron acceptor of PSI but not ferredoxin and ferredoxin-NADP-reductase (Forti and Meyer, 1969). Therefore the site of copper inhibition seems to be between the primary acceptor complex of PSI (see section 1.3.5) and NADP. The results of Table 8 indicate that the PSI component of the fast phase of absorption increase at 515 is inhibited by copper and the above mechanism might explain such data. Since copper has been already suggested to act as an external exciton quencher in the PSII pigments it is possible that the metal might induce similar effects in the PSI collector pigments. However this conclusion can only be suggested from the 515 absorption change studies and not proven as a definite effect.

In conclusion it has been shown that copper inhibits at a number of sites of the light reactions; at PSII, PSI and also possibly at the coupling factor complex. The studies on PSII activity suggest that copper acts as a quencher of the exciton energy in the light harvesting pigments but other possible mechanisms for inhibition at this site cannot be ruled out. A greater depth of understanding of the sites of inhibition has again been hampered by the lack of permeability of the whole Chlorella cells to various compounds which are used to isolate various components of the electron transport chain and energy conserving sites.

4.2 Mercury

4.2.1 Results

The experiments carried out using the other heavy metal ions Hg^{2+} , Pb^{2+} and Zn^{2+} were all under the same conditions as employed for the copper ions and therefore the technical aspects are not described unless different from the copper experiments.

4.2.1.1 The Effect of Mercury on steady state oxygen evolution

To determine the effects of mercuric ions on overall photosynthetic electron transport from the water splitting complex to the dark carbon fixation cycle, the effect of varying mercuric chloride concentrations was investigated on oxygen evolution using CO_2 as the terminal electron acceptor. The results are shown in Figure 55. They show that mercury inhibits oxygen evolution at a similar concentration to copper at about $1.5 \times 10^{-4}\text{M}$ as also reported by Kamp-Nielsen (1971). The concentration range over which mercury inhibits is however much narrower. Like the copper inhibition the effects of mercury are rapid and therefore different from the more long term effects seen on growth and cell division reported by Stratton et al. (1979) and Kamp-Nielsen (1971). Furthermore, no precipitate problems were encountered with higher concentrations of mercury (10^{-3}M) as had been found with copper.

4.2.1.2 The Effect of Mercury on chlorophyll fluorescence in the presence of DCMU

In order to investigate the effects of mercury on the photochemistry of PSII the effect of mercury on the fluorescence induction kinetics was investigated in a similar manner to the copper investigation. The results are shown in Figure 56 and indicate that the F_0 and F_m levels are quenched but a difference is seen from the copper data in the effect on the $(F_m - F_0)/F_m$ ratio. Although the data is not particularly precise it does show that mercury has no marked effect on the ratio.

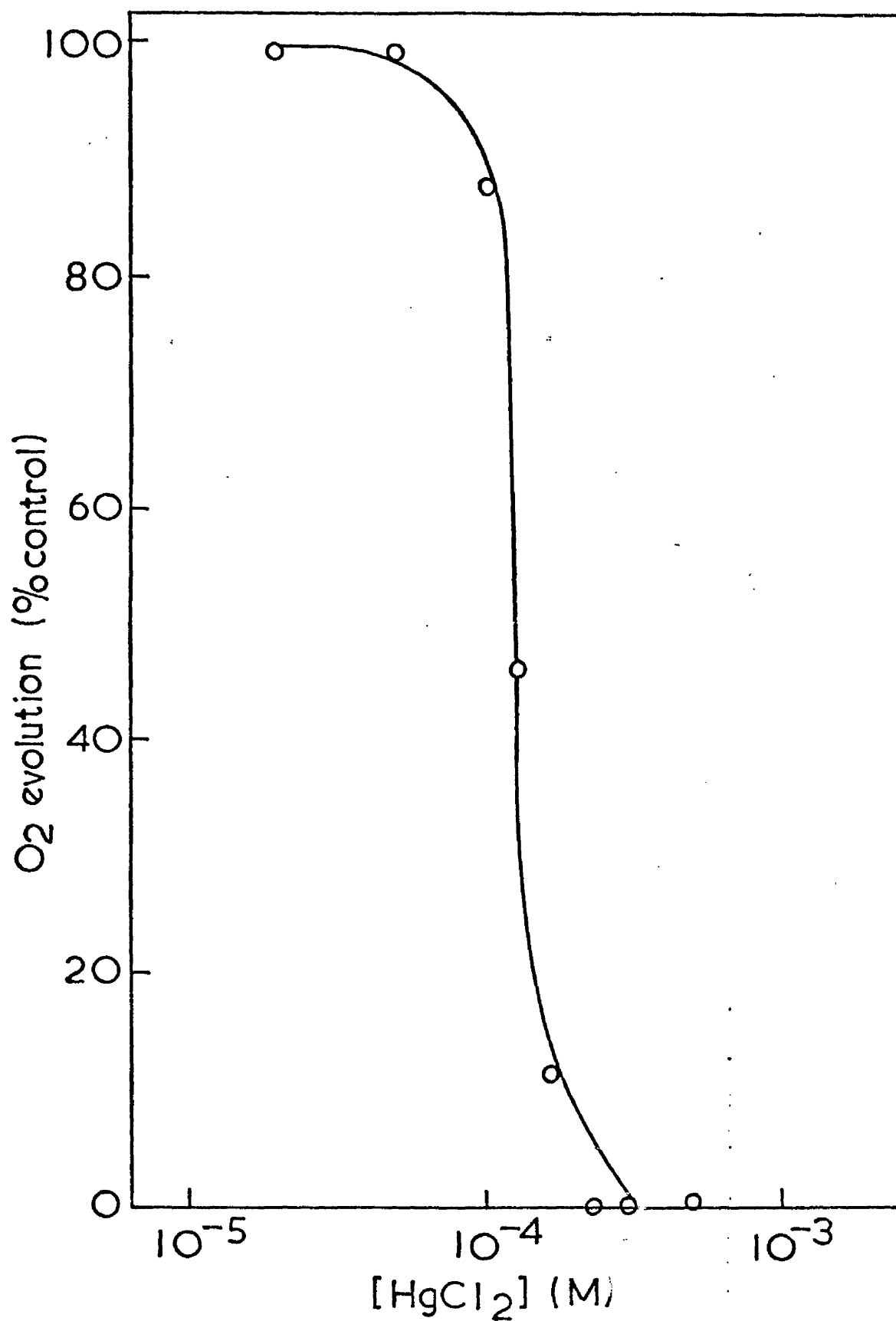


Figure 55. The effect of increasing concentrations of HgCl_2 on the steady state evolution of oxygen in Chlorella.

The control rate was $296 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

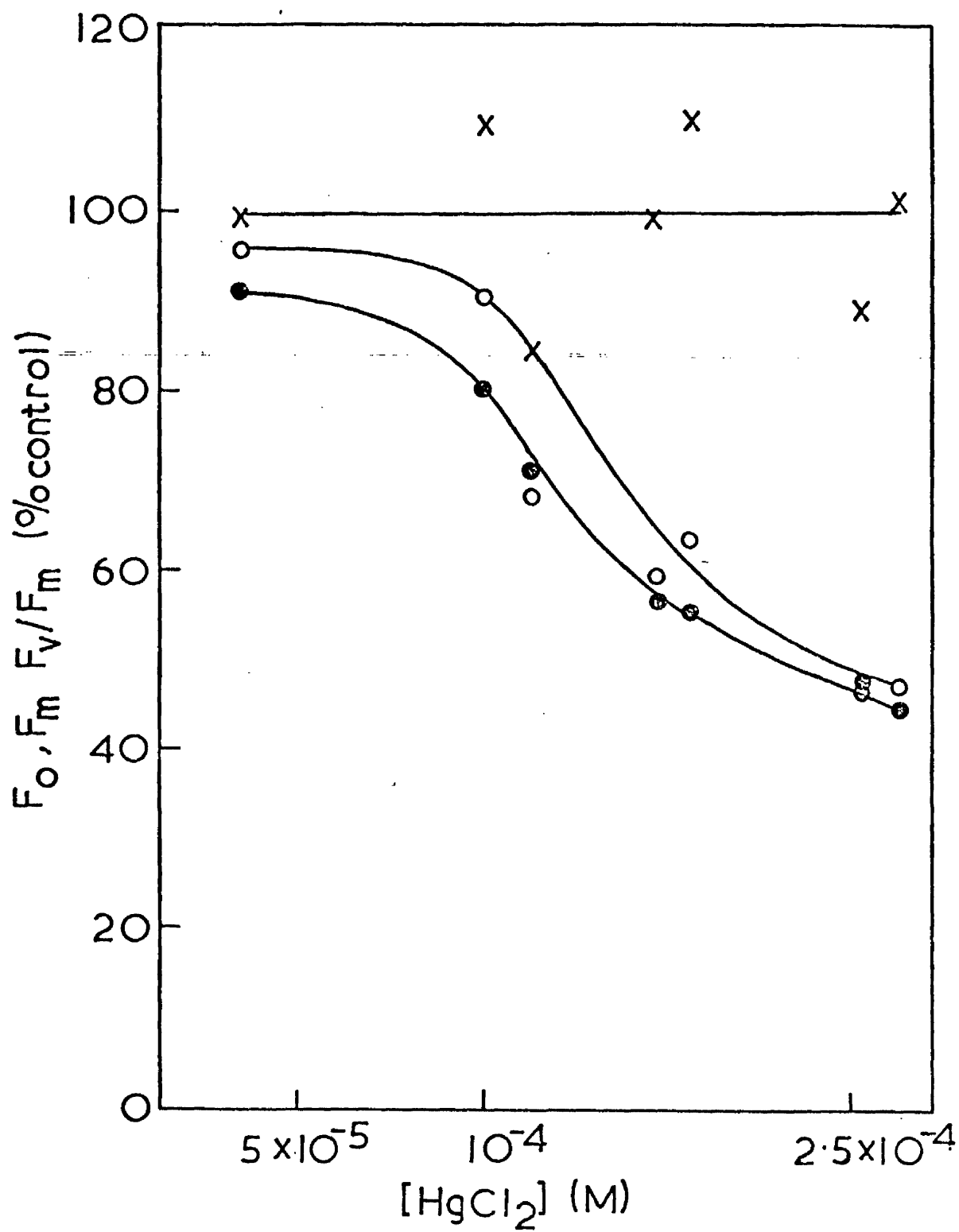


Figure 56. The effect of increasing concentrations of HgCl_2 on the chlorophyll fluorescence induction parameters F_0 (●), F_m (○) and F_v/F_m (x) in Chlorella after 5 minutes dark adaption in the presence of $10 \mu\text{M}$ DCMU.

This might be unexpected since quenching of both the F_0 and F_m levels suggest that mercury acts as an external quencher in the light harvesting collector pigments as described for the action of copper.

Figure 56 shows furthermore that F_m and F_0 levels are decreased by a similar proportion over the concentration range already found to inhibit steady state oxygen evolution, i.e. $1 - 3 \times 10^{-4}M$. Several reports have been made on the action of mercuric chloride on fluorescence in the presence of DCMU in isolated chloroplasts, Miles et al. (1973) found that concentrations of mercury greater than $10^{-4}M$ decreased both the F_0 and F_m levels of fluorescence although the F_m level was more markedly quenched. Similar results were shown by Kimimura and Katoh (1972). Miles et al. (1973) interpreted their results by suggesting that mercuric chloride accepts electrons from Q before the DCMU block, whereas Kimimura and Katoh (1972) presumed that the quenching was caused by effects on the pigment system or the reaction centre of the photosystem. My results would tend to support the latter hypothesis since the quenching of F_0 suggests that excited chlorophyll pigments are being de-excited before the energy has reached the photochemical reaction centre (see section 1.5.2.3).

4.2.1.3 The Effect of Mercury on Chlorophyll Fluorescence in the Absence of DCMU

Since the effects of mercury on the fluorescence induction in the presence of DCMU suggested a complex inhibition mechanism, it was decided further to investigate the effect of mercury on the overall movement of electrons through PSII by studying the effect of the metal on the fluorescence induction kinetics without DCMU. As discussed in section 4.1.1.4 the lack of DCMU allows electrons to move from Q to the inter-system electron transport components and consequently, since the fluorescence yield is generally governed by the redox state of Q, this fluorescence will indicate relative rates of electron transport to and from Q.

In addition, the yield will also be sensitive to the photochemical events of light harvesting and energy transfer such as changes in energy transfer to PSI (Bradbury and Baker, 1980) and radiationless de-excitation of the excited light harvesting pigments.

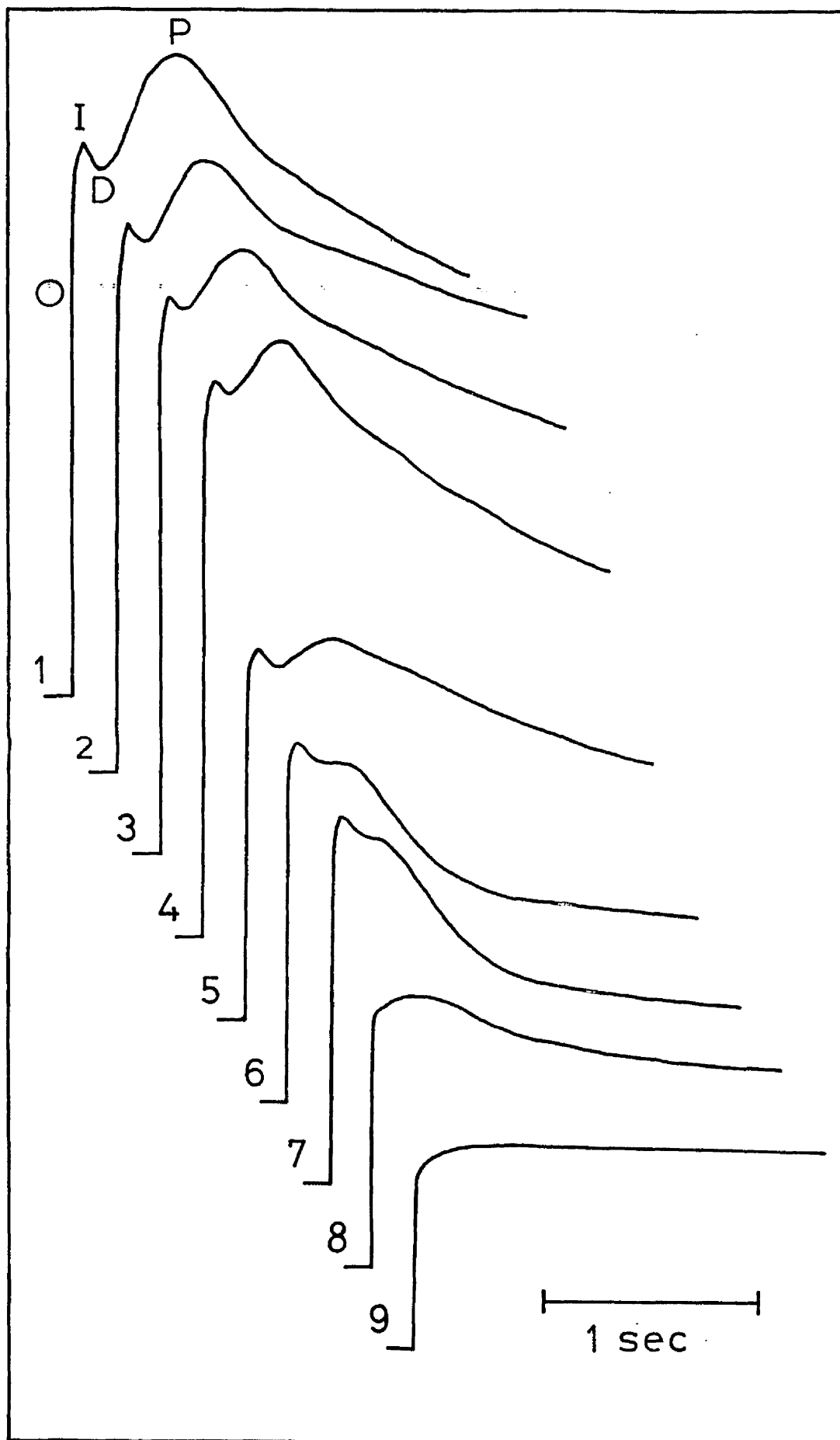
Figure 57 shows the fluorescence induction of Chlorella without DCMU in the presence of increasing mercuric chloride concentrations after 5 minutes dark incubation. Like copper the mercuric effects are complex depending on concentration and three main concentration dependent effects are seen; firstly at a concentration greater than 10^{-4} M, i.e. similar concentrations to those which inhibit oxygen evolution, a marked quenching of the fast rise was induced. Secondly over a similar concentration range the D - P rise is increasingly inhibited, and thirdly at high concentrations (greater than 2.0×10^{-4} M $HgCl_2$) the O - I peak is inhibited. Therefore mercury does not only act in a simple quenching manner as already defined in section 4.1.2 but also in a number of other ways. Since the D - P rise is inhibited it is suggested that one of the effects of mercury is either to inhibit electron flow to Q from the H_2O splitting complex via the PSII reaction centre or to increase the rate of reoxidation of Q via electron transfer to a secondary acceptor. This conclusion is also reached from the inhibition of the O - I rise which is thought to indicate the initial photochemical reduction of Q by PSII before it is reoxidised by the pool of intersystem carriers (Papageorgiou, 1975). Unlike the copper effects the I - D - P dip does not seem to be directly affected by mercuric ions.

4.2.1.4 The Effect of Mercury on Flash-Induced Oxygen Evolution

One of the major possibilities for the mechanism of mercury toxicity was suggested by it decreasing the rate of electron transfer from the water splitting complex to Q via the PSII reaction centre.

Figure 57. The effect of increasing concentrations of HgCl_2 on the chlorophyll fluorescence induction in the absence of DCMU (Kautsky phenomena) in Chlorella after 5 minutes dark adaption. Final concentrations of HgCl_2 were; 1, no addition; 2, $1.6 \times 10^{-5}\text{M}$; 3, $3.0 \times 10^{-5}\text{M}$; 4, $1.0 \times 10^{-4}\text{M}$; 5, $1.3 \times 10^{-4}\text{M}$; 6, $1.6 \times 10^{-4}\text{M}$; 7, $2.0 \times 10^{-4}\text{M}$; 8, $2.7 \times 10^{-4}\text{M}$; 9, $3.3 \times 10^{-4}\text{M}$.

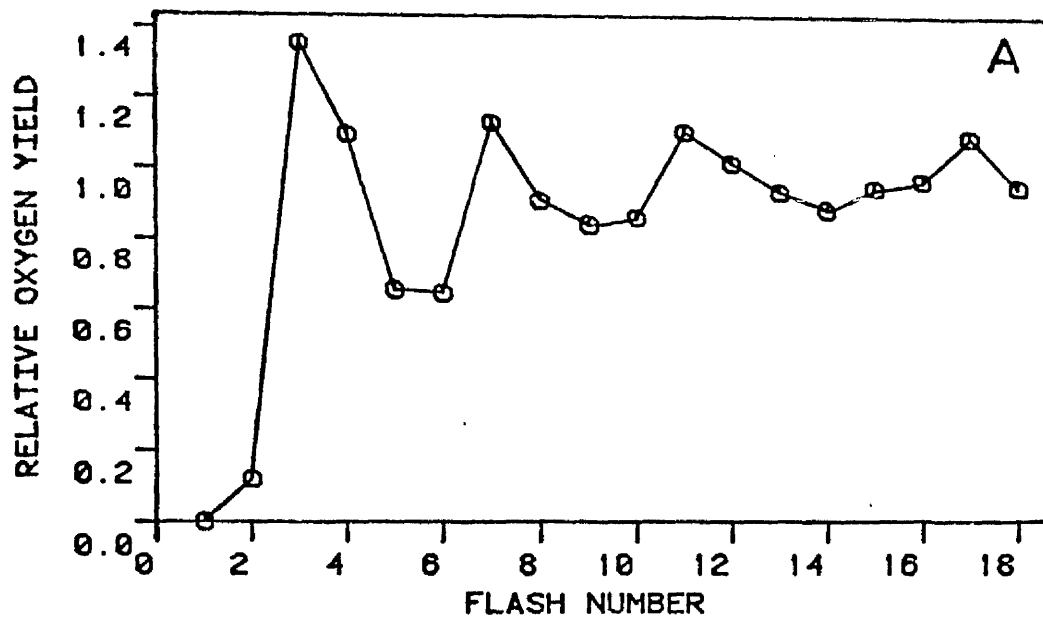
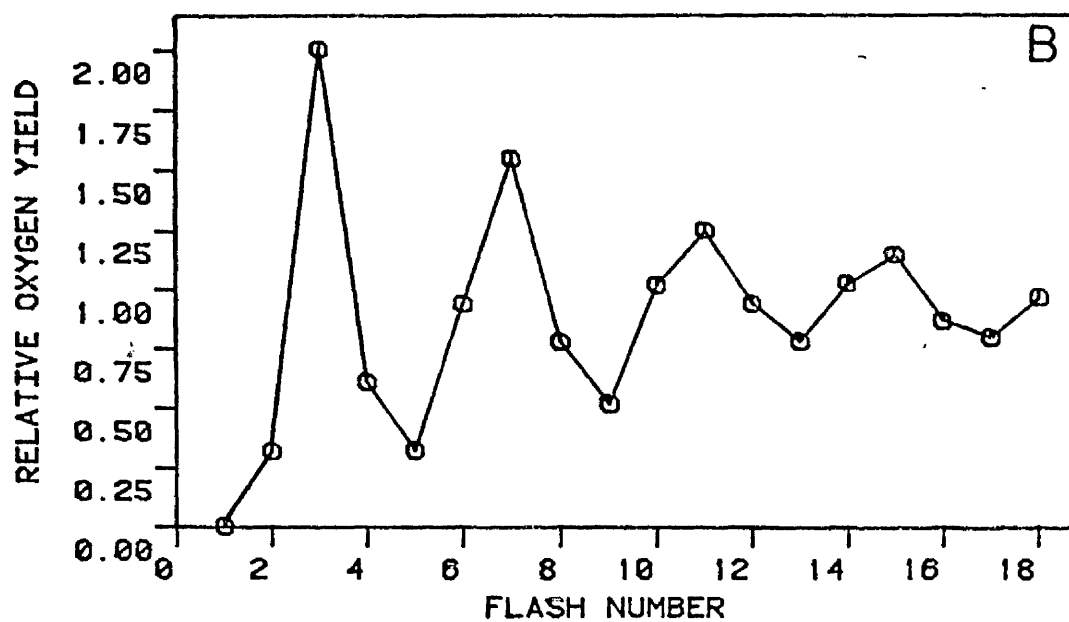
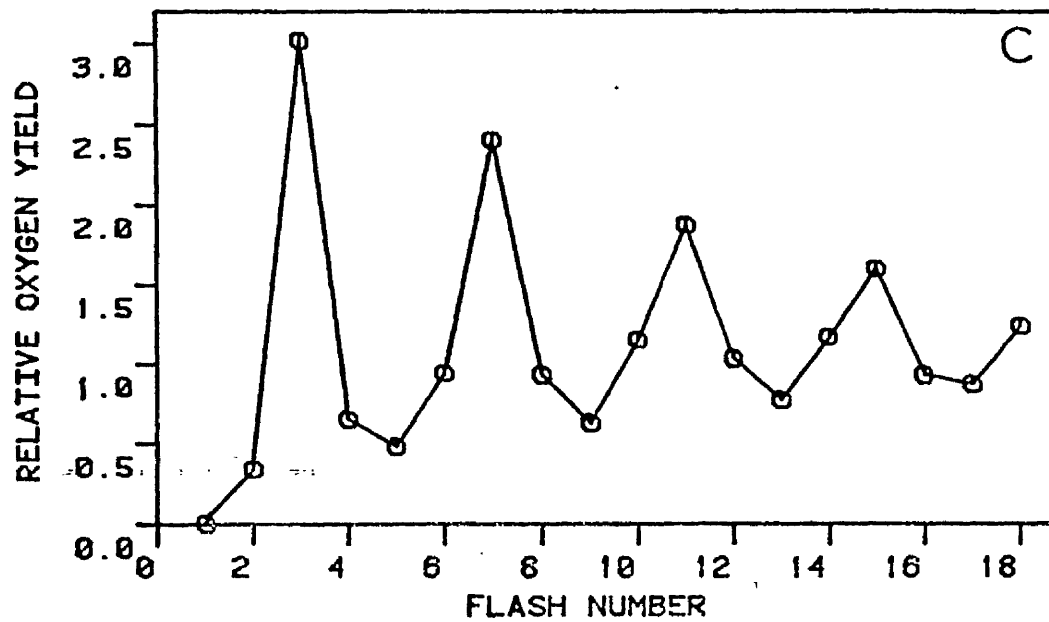
relative fluorescence

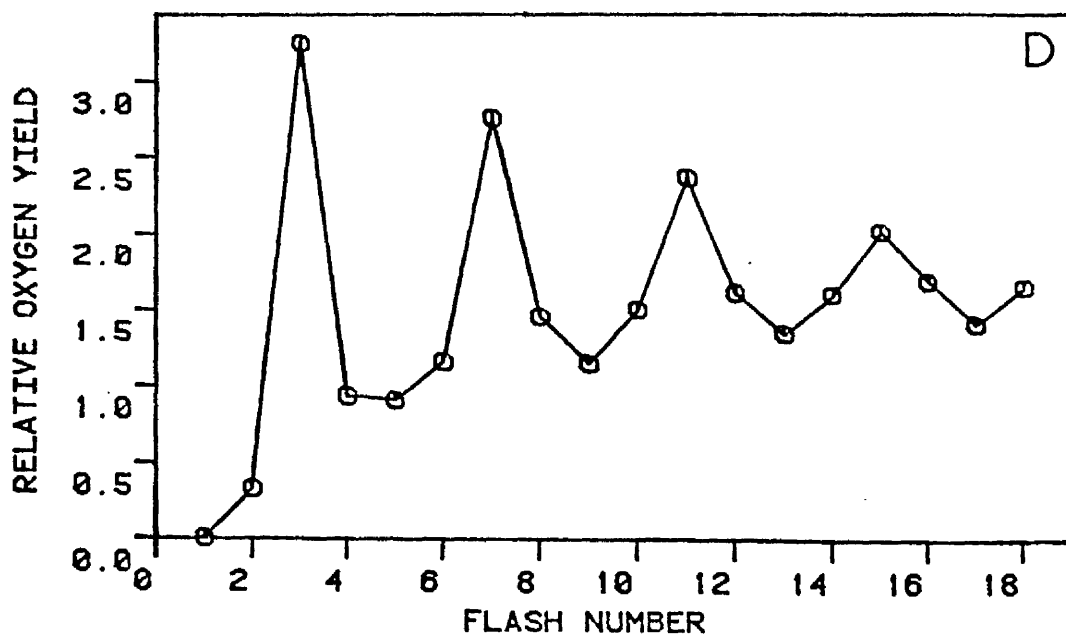
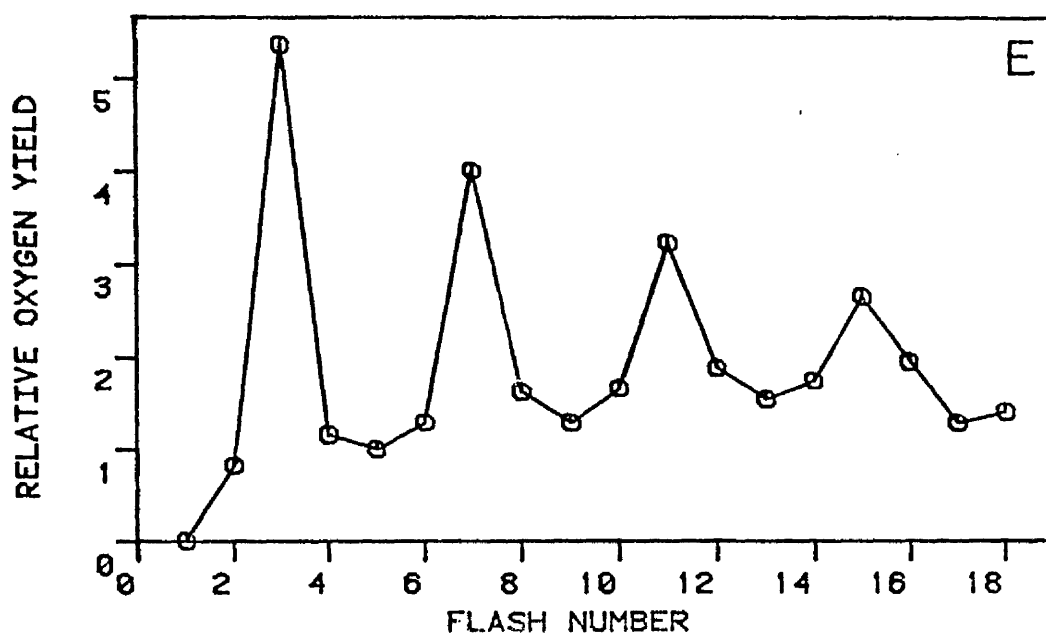
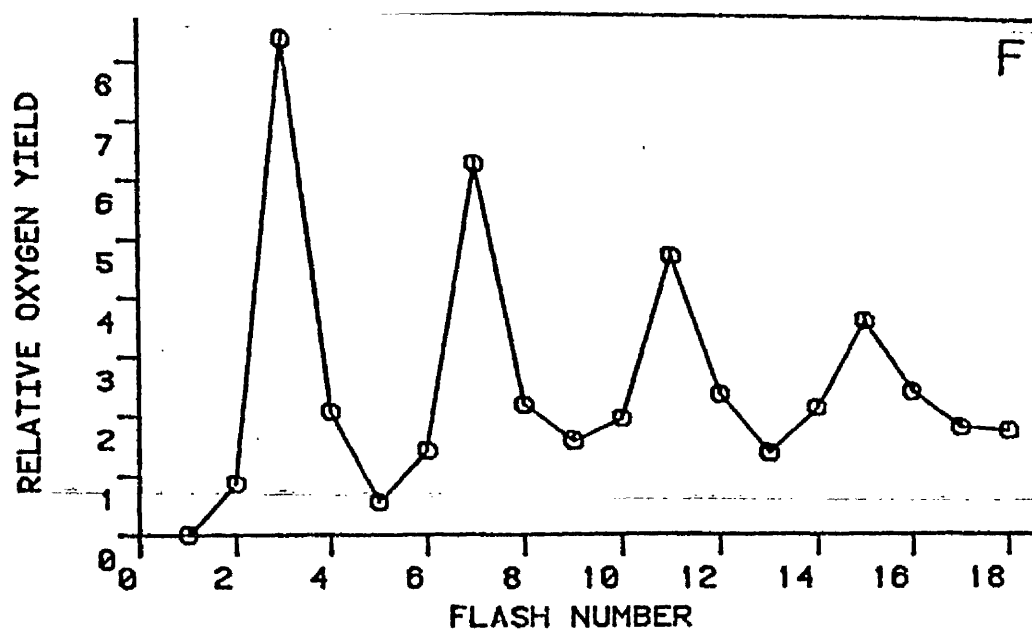


Therefore there was good evidence to suppose that mercury might be seen to interfere with the mechanism of H_2O splitting. Therefore the effects of increasing concentrations of mercuric chloride were investigated in a similar manner to copper on the oxygen yield pattern induced by a train of short flashes. As described in section 4.1.1.4 the algae were incubated with the mercury solutions for five minutes, then the cells were washed of the mercury solution and resuspended in buffer to prevent electrolysis and consequent artifacts caused by mercury deposition on the platinum electrode. As noted previously (section 4.1.1.5) the actual extents of the oxygen yields cannot be quantitatively compared since different samples are placed on the electrode on each occasion necessitating the disassembly of the electrode which can alter the gain of the electrode each time a new sample is used. Therefore only a qualitative analysis of the resulting flash-induced yield patterns may be obtained. The oxygen yields are presented as a fraction of the steady state value determined as the mean yield value of the 27th to 34th flashes and thus some measure of the steady state yield may be made with respect to the first twenty or so flashes.

Figure 58 shows the effect of increasing concentrations of mercuric chloride on the yield of oxygen induced by a series of flashes, after five minutes of dark incubation followed by the washing procedure. The data show a rather marked effect, namely a large decrease in the damping of the flash pattern with respect to the control. Furthermore the steady state yield of oxygen, although not a quantitative value, may be seen to be markedly decreased with respect to the yields of the first twenty flashes. As discussed in section 1.5.1.1 the yields of oxygen produced by short flashes occur in a distinct pattern, maximal on the third flash with a damped oscillation with a period of four. According to the theory adopted by Kok and coworkers (Kok et al. 1970) these phenomena represent the four positive charge accumulating 'S' states of the water splitting mechanism,

Figure 58. The effect of increasing concentrations of HgCl_2 on the yields of oxygen produced by a series of short saturating flashes, spaced 250 ms apart, in Chlorella after 5 minutes dark adaption. Relative oxygen yields represented as the yield per flash (Y_n) divided by the steady state yield (Y_{ss}) derived from the mean value of the 27th to 34th flash yields. A, no additions; B, with the addition of 10^{-4}M HgCl_2 ; C, with the addition of $1.35 \times 10^{-4}\text{M}$ HgCl_2 ; D, with the addition of $1.7 \times 10^{-4}\text{M}$ HgCl_2 ; E, with the addition of $2.0 \times 10^{-4}\text{M}$ HgCl_2 ; F, with the addition of $3.3 \times 10^{-4}\text{M}$ HgCl_2 .





which under ideal conditions would only yield a molecule of oxygen on the third, seventh, eleventh etc flash as the complex accumulated the four positive charges necessary for the splitting of two water molecules. However this perfect situation is not normally found and the periodicity of four is relatively quickly damped in the region of twenty to thirty flashes depending on the photosynthetic material used (Chlorella seem to show a more marked damping than do chloroplasts). Kok suggested that this damping was caused by an increasing degree of disorder brought about by some centres being inactive (misses) and some carrying out the reaction twice (double hits). However although the "double hits" theory may be substantiated by shortening the length of the flash (Joliot et al. 1971) the "misses" theory of damping resulting from some photochemical centres being inactive has been questioned in recent years (e.g. Lavelle, 1978). This results from the assumption that if photochemical misses increase due to inactive centres then the steady state yield of oxygen should decrease since less oxygen will be evolved per quanta absorbed. Although such an assumption has been found in some cases e.g. where no electron acceptor is available to accept electrons from Q in isolated chloroplasts (Radmer and Kok, 1972), a number of treatments have been reported which imply that both misses and the steady state yield may be decreased in parallel. The data shown in Figure 58 is in general agreement with this latter suggestion and consequently a different type of inhibitory mechanism is discussed in association with the fluorescence data.

4.2.1.5 The Effect of Mercury on the Absorption Change at 515nm

As mercury has been reported to act as an energy transfer inhibitor (Izawa and Good, 1969) and has also been shown possibly to interfere with the light harvesting pigments by acting as an external quencher, it was decided to investigate the effect of mercuric chloride on the field indicating absorption change at 515nm.

The algae were incubated for five minutes in the dark with various concentrations of the metal salt under similar conditions to those in section 4.1.1.6. It should be noted that a similar experiment was carried out with mercuric chloride in section 3.1.8 (see Figure 34) but the two studies were carried out under different experimental conditions, i.e. chlorophyll concentration, incubation time, etc., so it was deemed necessary to repeat the effect of mercury on the 515 change under the same conditions as employed for the rest of the heavy metal studies. Figure 59 shows the effects of increasing mercury concentrations on the extent of the fast phase, slow phase and overall rate of decay of the absorption change at 515nm, the interpretation of which has already been described (see section 1.5.3). The data show a similar effect to that found with copper and the classical uncouplers as well as mercury itself under the different conditions of the previous chapter (see Figure 34). That is, the rate of decay is markedly decreased over a narrow concentration range at which oxygen evolution is inhibited, i.e. $1.0 - 2.5 \times 10^{-4}M$ and over the same concentration range the extent of the slow phase is increased.

A control trace and a typical trace, showing the increased extent of the slow phase and decreased rate of decay brought about by the addition of $1.5 \times 10^{-4}M$ $HgCl_2$, were deconvoluted in a similar manner to the studies using uncouplers and $CuSO_4$ (see section 3.1.10). The data are shown in Table 8. Like these other compounds $HgCl_2$ was found to stimulate the extent of the slow phase and to markedly decrease the rate of decay. Like copper, but unlike the classical uncouplers the extent of the fast phase is decreased at concentrations greater than $10^{-4}M$, where oxygen evolution is inhibited, suggesting that the primary charge separation at the reaction centres is inhibited by mercury. Thus mercury may be seen to act in an uncoupling mode akin to the phenolic uncouplers and copper.

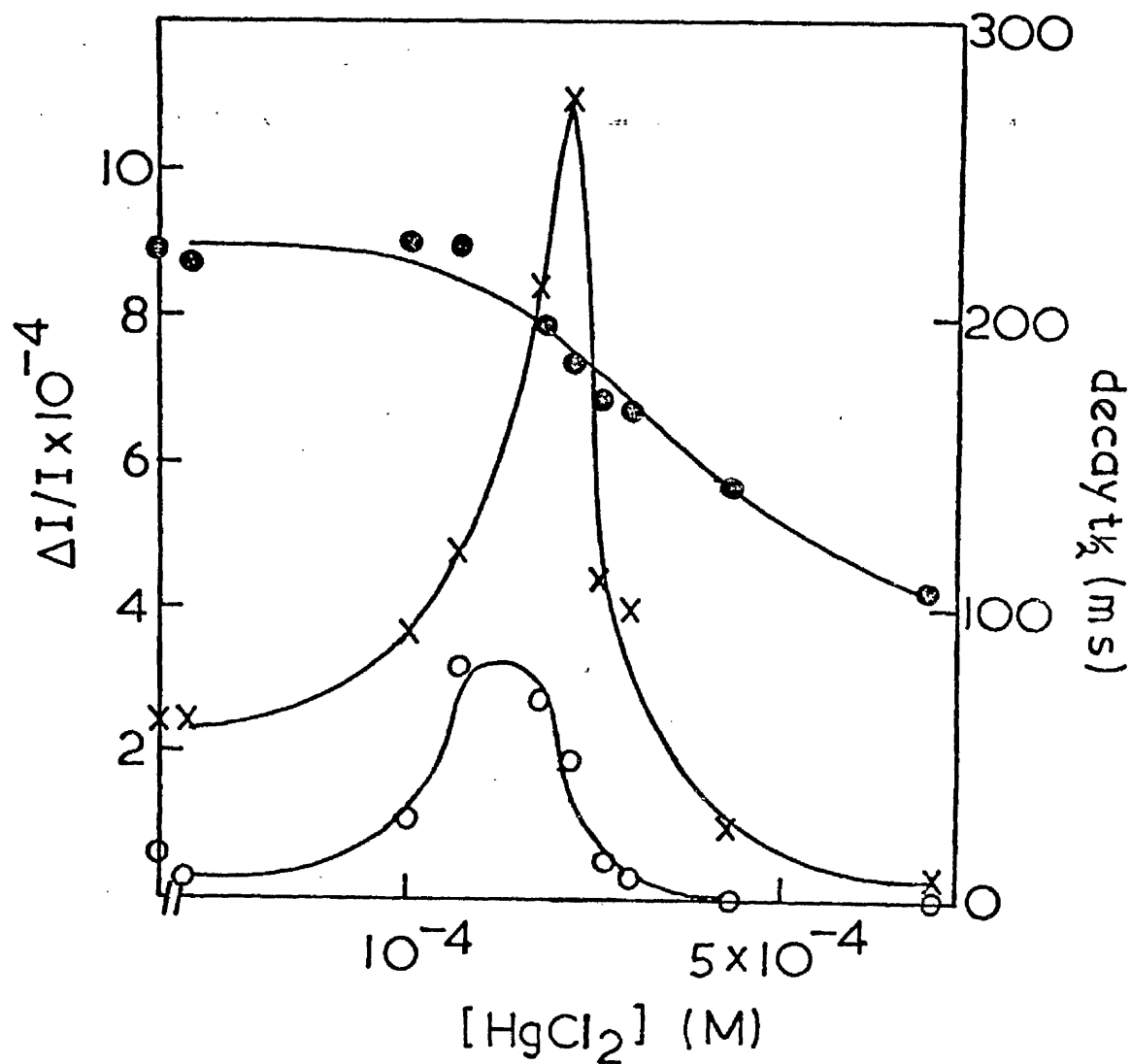


Figure 59. The effect of increasing concentrations of HgCl_2 on the fast phase (\bullet), slow phase (o) and the decay half-time (x) of the flash-induced absorption change at 515 nm in *Chlorella* after 5 minutes dark incubation. Each point represents the accumulated average of 128 sweeps at a flash frequency of 0.5 Hz.

Table 8

Deconvolution of Typical Experimental Traces of the Flash-Induced
Electrochromic Absorption Change under (a) Control Conditions and (b)
in the Presence of $1.5 \times 10^{-4}\text{M}$ HgCl_2 , According to a Linear Combination
of Two Exponentials: $\Delta A(t) = \{ A + B(1 - \exp -kt) \} \exp -lt.$

Treatment	$A(\Delta I/I \times 10^{-5})$	$B(\Delta I/I \times 10^{-5})$	$k(\text{msec})$	$l(\text{msec})$
(a) Control	10.0 ± 1.29	1.34 ± 0.58	2.54 ± 2.12	50.70 ± 2.12
(b) + $1.5 \times 10^{-4}\text{M}$ HgCl_2	7.97 ± 0.45	2.65 ± 0.20	* 10.55 ± 3.66	239.67 ± 26.64
(b)/(a) x 100%	79.7	197.6	415.35	472.72

* Footnote to Table 8. It should be noted that the half-rise time of the slow phase is increased by $1.5 \times 10^{-4}\text{M}$ HgCl_2 .

High concentrations of mercury show the typical uncoupling effects of a markedly increased rate of decay arising from the increased rate of dissipation of the high energy intermediate because of the greater ionic permeability of the thylakoid membrane. The decrease in the rate of decay and increase in extent of the slow phase of increase at low mercury concentrations may be explained in a similar manner to that described in the copper and CCCP discussions, i.e. that the mercury decreases the rate of decay of the absorption change which allows the slow phase to be more easily seen or that it interferes with the field controlling the redox poise of the components responsible for the slow electrogenic increase.

The decreasing extent of the fast phase at the same concentrations as those which stimulate the slow phase extent and decrease the rate of decay suggests that a second effect is superimposed, namely an inhibition of the primary charge separation reactions at the reaction centres. This was further investigated in a similar manner to the copper experiments by blocking the reactions of PSII with DCMU and NH_2OH . The data are shown for a number of mercury concentrations in Table 9 and show that the same effect as copper is found, namely that the fast phase of increase is increasingly inhibited, to an almost total degree when PSII is blocked, by increasing mercury concentrations. This suggests that the primary reactions of PSI are markedly inhibited by mercury.

4.2.2 Discussion

The experiments conducted on the light reactions of photosynthesis in the presence of concentrations of mercuric chloride in the range of circa $10^{-5} - 10^{-3}\text{M}$ have shown that the inhibitory effects of this heavy metal are complex and do not result from a single inhibitory mechanism.

Table 9

The Effect of Increasing Concentration of HgCl₂ on the Fast Phase of Absorption Increase at 515nm in the Presence and Absence of 10 μ M DCMU and 100 μ M NH₂OH

[HgCl ₂] (M)	Extent of Fast Phase of Δ 515			
	No Addition		+ 10 μ M DCMU, 100 μ M NH ₂ OH	
	$\Delta I/I \times 10^{-5}$	% Control	$\Delta I/I \times 10^{-5}$	% Control
Control	7.54	100	5.22	69.2
+ 5.0 x 10 ⁻⁵	7.27	96.4	4.95	65.6
+ 1.0 x 10 ⁻⁴	7.25	96.1	4.49	59.5
+ 1.3 x 10 ⁻⁴	5.22	69.2	3.03	40.2
+ 1.7 x 10 ⁻⁴	5.15	68.3	1.29	17.1
+ 2.0 x 10 ⁻⁴	4.60	61.0	0	0

The algae were incubated for 5 minutes in the dark with the various HgCl₂ concentrations, after 10 seconds pre-illumination, (2 x Schott RG 680 red filters, giving an intensity of 60Wm⁻²) in the absence or presence of 10 μ M DCMU and 100 μ M NH₂OH. Each trace represented the accumulated average of 128 sweeps at a flash frequency of 0.5 Hz.

Since the inhibitions were found after only a relatively short incubation of approximately five minutes the inhibition results from a direct immediate effect on the light reactions rather than from the slow effects seen on growth and cell division or other physiological processes (see Stratton et al. 1979).

To determine the possible sites of action of the heavy metal the individual effects on fluorescence, flash-induced and steady state oxygen evolution and the field indicating absorption at 515nm are considered bearing in mind possible connections between these effects which might arise from mercury affecting the same components or site. In all cases, both under single turnover and steady state conditions mercuric chloride inhibited specifically in the concentration range from 1.0×10^{-4} to 3.0×10^{-4} M when algae of $5\mu\text{g ml}^{-1}$ chlorophyll were used. However in increasing the concentration of mercuric chloride in this range different effects predominated at different concentrations.

The data presented here suggest that mercury may act at four different sites; (a) the light harvesting pigments and reaction centre of photosystem II and its photochemical reactions, (b) the water splitting complex, (c) the charge separation at PSI and (d) the movement of ions through the membrane possibly at the coupling factor. These four possible sites of action are now considered.

Increasing concentrations of mercury have been shown to quench the F_0 and F_m levels of fluorescence but not the ratio F_v/F_m (Figure 56). Furthermore one of the effects of mercury on the fluorescence transients without DCMU is an overall quenching of the size of the emission. Since the F_0 level is decreased then the first mode of mercury toxicity may be as a classical external quencher in a manner akin to copper (see section 4.1.2) and dinitrobenzene (Etienne et al. 1974).

Since, neither changes in energy transfer between the two photosystems, nor changes in radiationless deactivation of the excited chlorophyll pigments (rate constant k_t and k_h respectively) can be distinguished in the experiments carried out here, a general rate constant for an extra pathway of exciton dissipation, k_q , should be included in the equations representing the F_o , F_m and F_v/F_m levels of fluorescence (see section 4.1.2). The addition of this extra pathway for energy dissipation should result in the F_o , F_m and F_v/F_m levels decreasing with increased mercury concentration as shown by copper. However since the F_v/F_m level, although rather variable, remained relatively constant on incubation with increasing concentrations of mercury then modification of another rate constant is predicted if the proposal of mercury acting as an external quencher is to be maintained (see Butler and Kitajima, 1975). In order to accommodate the proposed quenching of chlorophyll fluorescence by mercury into the equations of section 4.1.2 it is proposed that, in addition to external quenching, mercury decreases the magnitude of the yield of non-radiative decay at the reaction centre. It can be seen that by adding k_q to the three equations of section 4.1.2 they would all decrease, but if the magnitude of k_T [ød] was also decreased in the F_m and F_v/F_m expressions then the decrease in F_m would be limited and the F_v/F_m value maintained whilst the F_o value would still be quenched. So, although, by decreasing the F_o values, mercury is exhibiting a classically external quenching behaviour an extra effect of decreasing the yield of radiationless de-excitation at the reaction centre has been assumed to accommodate the expressions of fluorescence analysis as in section 4.1.2.

The exact nature of the non-radiative decay at the reaction centre is not well defined although several mechanisms have been proposed; Haehnel et al. (1982) have suggested that the loss of energy at the closed reaction centre may result from charge recombination between the oxidised reaction centre chlorophyll and the transiently reduced primary electron acceptor (possibly a pheophytin molecule) resulting in the production of ground or triplet state chlorophyll molecules. One possible method by which mercury might exert such effects is that reported by Miles et al. (1973) who presented evidence that at 10^{-4} M, mercuric chloride inhibits electron transport and fluorescence in the presence of DCMU by acting as an electron acceptor from Q, or in place of it, in isolated chloroplasts. This would prevent the charge recombination at the closed reaction centre by competing for the electrons at the transiently reduced primary acceptor.

This mechanism of mercury accepting electrons from Q might also account for the second type of action of mercury, illustrated as a decrease in the D - P rise of the fluorescence traces measured in the absence of DCMU. The D - P rise may be attributed to the rereduction of Q after the intersystem pool of electron carriers has been reduced and consequently if mercury acted by accepting electrons from Q as proposed by Miles et al. (1973) then the D - P rise would be inhibited since Q would not attain its transiently rereduced state. The decreasing level of F_m and the D - P rise would seem to concur with this conclusion particularly since at relatively high concentrations the effect may be seen to be so marked as to prevent the I level of initial photochemical reduction of Q being attained. However inhibition of the "Kautsky" induction may arise not only from increased electron flow away from Q but from decreased electron flow to it from the donor side of PSII.

Therefore the decrease of the D - P rise and F_m levels of fluorescence may equally be interpreted as an inhibition on the donor side of PSII or at the water splitting complex. This site has been shown to be particularly sensitive to heavy metals (see section 1.6). Increasing inhibition of the water splitting mechanism would be expected to decrease the I - D - P levels of Kautsky fluorescence, as shown in Figure 57 and might also be expected to cause changes in the flash-induced oxygen evolution transients by the water splitting complex. The data of Figure 58 does indeed show that mercury markedly modifies the flash-induced oxygen sequence and thus suggests that this is a site of toxicity of the metal.

Although the experimental procedure precluded a quantitative comparison of flash-induced oxygen yield magnitudes, the steady state yield is seen to decrease markedly with respect to the first twenty flashes and it can be concluded that either the steady state oxygen yield is increasingly inhibited by increasing mercury concentrations or that the oxygen evolved by the first few turnovers of the water splitting complex is markedly increased. The inhibition of steady state oxygen evolution (Figure 55) at similar concentrations tends to support the former conclusion that mercury inhibits the evolution of oxygen by the water splitting complex. In addition to the decreasing steady state oxygen evolution levels a marked decrease in the damping is seen. The original interpretation of Kok (Kok et al. 1970) assumes that the water splitting complexes are independent, non-diffusible centres and consequently inhibition of the centres should only decrease the extent of the yields and not alter the flash pattern. Alteration of the pattern should thus only result from anomalies in the photochemical centres such as double hits or misses (see section (1.5.1.1)).

A decreasing steady state yield would thus be expected to be due to increased numbers of inactive centres (misses) which would cause increased damping of the oscillation. The data of Figure 58 do not concur with this antiparallel correlation between damping and steady state yields. Maison and Lavorel (1977) found that thermal deactivation of the centres decreased the steady state yield along with the damping. Similar observations were noted for the addition of p-benzoquinone (Diner and Joliot, 1976) sodium azide (Maison and Etienne, 1977) and CCCP (Etienne, 1974b). Lavorel (1976 and 1978) has suggested that such phenomena may be explained by assuming that there is no strict stoichiometry between the water splitting complexes (E) and the photochemical reaction centres (P^+). Originally he proposed that the water splitting centres are either free or bound, within the internal space of the thylakoid, to the donor side of P^+ (Lavorel, 1976). He later modified this by assuming that the water splitting complexes were intrinsic proteins (or protein complexes) embedded in the membrane, endowed with lateral motion due to the fluidity of the membrane (Lavorel, 1978). He then proposed that if the number of water splitting complexes (nE) became greater than the number of photochemical centres (nP^+) by inactivation of the photochemical complexes then the total number of water splitting complexes (nE) which received a positive charge from the photochemical complexes (nP^+) would contribute to oxygen evolution but not enough would be available for all nE . This would result in a maximal yield of oxygen per light absorbed ($Y_{ss} = nP^+$) but a number of the water splitting complexes would not receive positive charges ($nE - nP^+$) and this number of misses would occur. If the opposite conditions prevailed and nE was less than nP^+ almost every E would receive a positive charge from a P^+ and very few misses, and hence little damping, would occur.

Since a number of photochemically produced positive charges would not be used (and consequently would probably recombine with the negative charges) the yield of oxygen produced per flash absorbed would be decreased.

The data obtained here with mercury agrees with this latter interpretation that mercury inhibits the number of water splitting complexes. An inhibition by mercury at the water splitting complex site would also induce decreases in the fluorescence parameters, F_m and $I - D - P$. It is therefore suggested to be a probable site of mercury toxicity.

The third site of mercury inhibition proposed results from the inhibition of the fast phase of $\Delta 515$. Under conditions where PSII is blocked the primary charge separation due to PSI is shown to be almost totally inhibited (Table 9). The experiments carried out were not capable of elucidating the exact nature of this inhibition but a number of possible modes of inhibition may be postulated from the literature and the other data with mercury. Firstly since mercury has been proposed to quench excitation energy in the pigments of PSII, it is possible that a similar action may take place in the pigments of PSI. Quenching of excitation energy in the light harvesting pigments of PSI would decrease charge separation at the PSI reaction centre. Secondly Kimimura and Katoh (1972) have shown that mercury clearly inhibits the PSI donor plastocyanin and hence blocks PSI electron transport. The prevention of electron donation to PSI by mercury at the plastocyanin site would be expected to prevent the light reactions of PSI and so inhibit the extent of the primary charge separation measured at 515nm. Thirdly both ferredoxin and ferredoxin-NADP-reductase (see section 1.6.2) are inhibited by organic mercurials such as phenyl mercuric acetate and p-chloro-mercuribenzoate (Izawa and Good, 1972; Honeycutt and Krogmann, 1972) and it is possible that inorganic mercury may act at the same site. Copper has been shown to act at this site (Shioi et al. 1977) and considering the similarity between the effects of the two metals on the absorption change at 515nm this site may be suggested to be similarly sensitive to mercury too.

The final mode of action of mercury shown here is observed as a decrease in the rate of decay of the 515 absorption change and a stimulation of the slow phase. This is followed at higher concentrations by an increasing rate of decay and dissipation of the slow phase. This type of action has already been indicated for the phenolic uncouplers (see Chapter 3) and thus mercury may be suggested to be acting in an uncoupling manner. There do not seem to be previous reports of mercury acting as an uncoupler but a number of early reports do suggest that it acts as an energy transfer inhibitor possibly by blocking phosphorylation near the terminal steps of ATP synthesis (see section 1.6.2). The blocking of phosphorylation might be expected to decrease the rate of decay of the 515 absorption change (section 1.5.3.3) and in accordance with the proposals of Chapter 3 let the slow phase be more easily observed. However the increased rate of decay at higher concentrations suggests that the ionic permeability of the thylakoid membrane is increased in a manner akin to classical uncoupling. Therefore energy transfer inhibition alone cannot explain the action of mercury on $\Delta 515$ and it may be assumed that mercury increases the ionic permeability of the membrane either at the coupling factor itself or non specifically in a manner similar to uncouplers.

In conclusion it can be seen that there does not seem to be one specific site of mercury inhibition of the light reactions. A number of possible inhibitory mechanisms have been suggested to explain the data accumulated but no single one of these can be assumed to explain the effects of mercury seen. As already discussed in previous sections the isolation of specific sites of mercury action is hindered by the impermeability of the intact Chlorella cell to a number of compounds which make isolation of specific inhibitory sites in isolated chloroplasts much more partial.

Therefore although the isolated chloroplast is a system suffering from a number of possible experimental artifacts by the very nature of its isolation it is suggested that further studies on the toxicity of mercury be carried out either in chloroplasts isolated from higher plants or algal subcellular preparations. The possibility of using fractured algal preparations is initially an agreeable proposition but methods which break open the cell by pressure or sonication or enzymatic digestion may well cause damage to the chloroplast integrity itself and therefore may prove not to be so useful.

4.3 Lead

4.3.1 Results and Discussion

4.3.1.1 The Effect of Lead on Steady State Oxygen Evolution

In order to determine the inhibitory concentrations of lead on overall photosynthetic electron transport the effect of varying concentrations of $\text{Pb}(\text{NO}_3)_2$ on oxygen evolution using CO_2 as the terminal electron acceptor was studied under the conditions similar to those described previously (section 4.1.1.1). Figure 60 (curve a) shows the effect of increasing concentrations of lead on the oxygen evolution after 5 minutes incubation in the dark; the rate was taken 5 minutes after the sample was illuminated to allow a steady rate of oxygen production to develop. The data show that far higher concentrations of lead were needed to inhibit the oxygen evolution of Chlorella (circa $5.0 \times 10^{-3}\text{M}$) than copper or mercury. This is in agreement with the inhibitory concentrations for lead reported for isolated chloroplasts (Miles et al. 1971) and in whole leaf segments (Homer et al. 1980) although lower concentrations inhibited the oxygen evolution of the marine green alga Phaeodactylum tricornutum (Overnell, 1975a) suggesting a species difference.

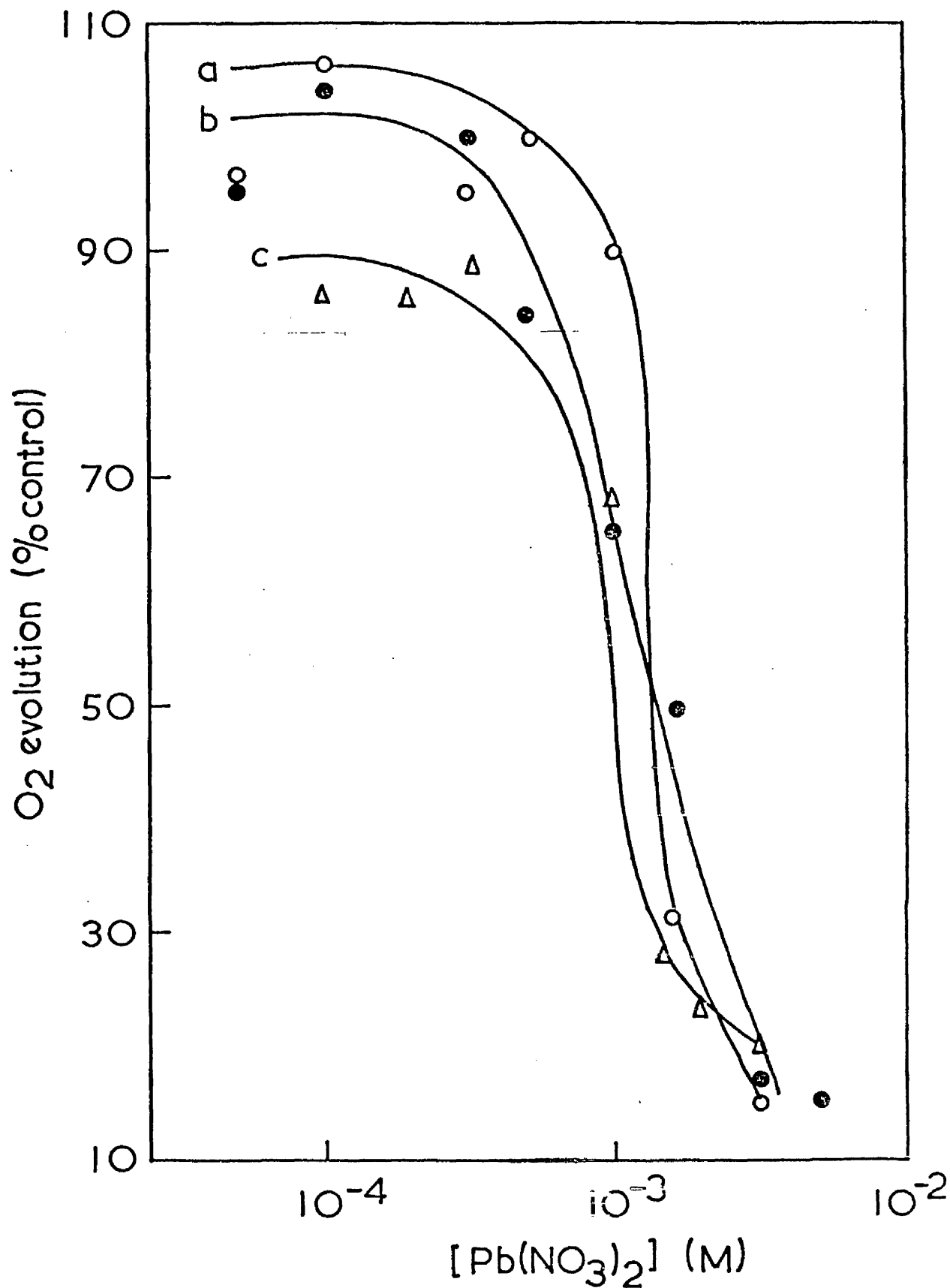


Figure 60. The effect of increasing concentrations of $\text{Pb}(\text{NO}_3)_2$ on the steady state evolution of oxygen in *Chlorella*. (o), after 5 minutes dark incubation, $5 \mu\text{g ml}^{-1}$ chlorophyll; (•), after 5 minutes light incubation, $5 \mu\text{g ml}^{-1}$ chlorophyll; (Δ), after 5 minutes dark incubation, $30 \mu\text{g ml}^{-1}$ chlorophyll.

The control rates were 212, 226 and $236 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$ for curves a, b and c respectively.

At the concentrations which inhibited oxygen evolution a precipitate appeared in the electrode reaction cell which suggested that a possible cause of the inhibition was due to lead binding to a compound in solution rather than an inhibitory effect at the thylakoid membrane itself. Since the pH titration curve of the MOPS buffer was not significantly affected by lead the precipitate was thought to be due to the lead complexing with the bicarbonate solution which was added as a carbon source. That this hypothesis was correct was suggested by further experiments on the lead inhibition of oxygen evolution.

Firstly, the effects of light incubation as opposed to dark incubation were investigated on the lead inhibitory concentrations of oxygen evolution. Curve b in Figure 60 shows that there was no significant difference between the light and dark incubations. Secondly the effect of a higher chlorophyll concentration ($30\mu\text{g ml}^{-1}$) was investigated on the lead inhibition. Curve c in Figure 60 also shows that the lead inhibition did not seem to depend on the chlorophyll concentration of the algal sample since no significant difference was observed between the concentrations of lead inhibition on algal chlorophyll concentrations of $5\mu\text{g ml}^{-1}$ and $30\mu\text{g ml}^{-1}$. Since the binding of metals to the inhibitory sites of the algae should be affected both by metal to chlorophyll concentration ratios and also by incubation conditions (Steeman-Nielsen et al. 1969) then the lead was thought to be affecting the carbon source bicarbonate in solution, before it could be used by the algae, and suggested that lead inhibits photosynthesis in Chlorella by rate limiting the dark fixation cycle by complexing its substrate.

This was investigated by varying the effect of bicarbonate concentration on inhibition of oxygen evolution by two lead concentrations 10^{-3}M and $5.0 \times 10^{-2}\text{M}$ which inhibited by circa 50% and 85% respectively under the standard experimental conditions of $5\mu\text{g ml}^{-1}$ chlorophyll concentration.

Figure 61 shows the results; the algae were incubated for five minutes in the dark with the lead concentrations or with no addition, the bicarbonate at various concentrations was then added and the rate of oxygen evolution measured after five minutes illumination to allow a steady rate of oxygen production to develop. The control trace shows that without any HCO_3^- very little oxygen evolution took place but when increasing concentrations were added the oxygen production increased to a maximum at about 10^{-3}M NaHCO_3 . When the two inhibitory concentrations of lead were added very little oxygen evolution occurred at low HCO_3^- conditions but as the HCO_3^- concentration was increased so the inhibition by lead was prevented. The larger concentration of lead required a larger HCO_3^- concentration to relieve the inhibition. These results support the hypothesis that lead inhibits the oxygen evolution of Chlorella by binding to the bicarbonate which acts as the carbon source for carbon fixation by the algae.

Therefore since the inhibition of lead appeared to be due to its binding of bicarbonate and not to a direct inhibition of the light reactions no further experiments were conducted.

4.4 Zinc

4.4.1 Results and Discussion

4.4.1.1 The Effect of Zinc on Steady State Oxygen Evolution

As in the investigations on the three other heavy metals the first experiments carried out with zinc ions were to determine at what concentrations the metal inhibited photosynthesis.

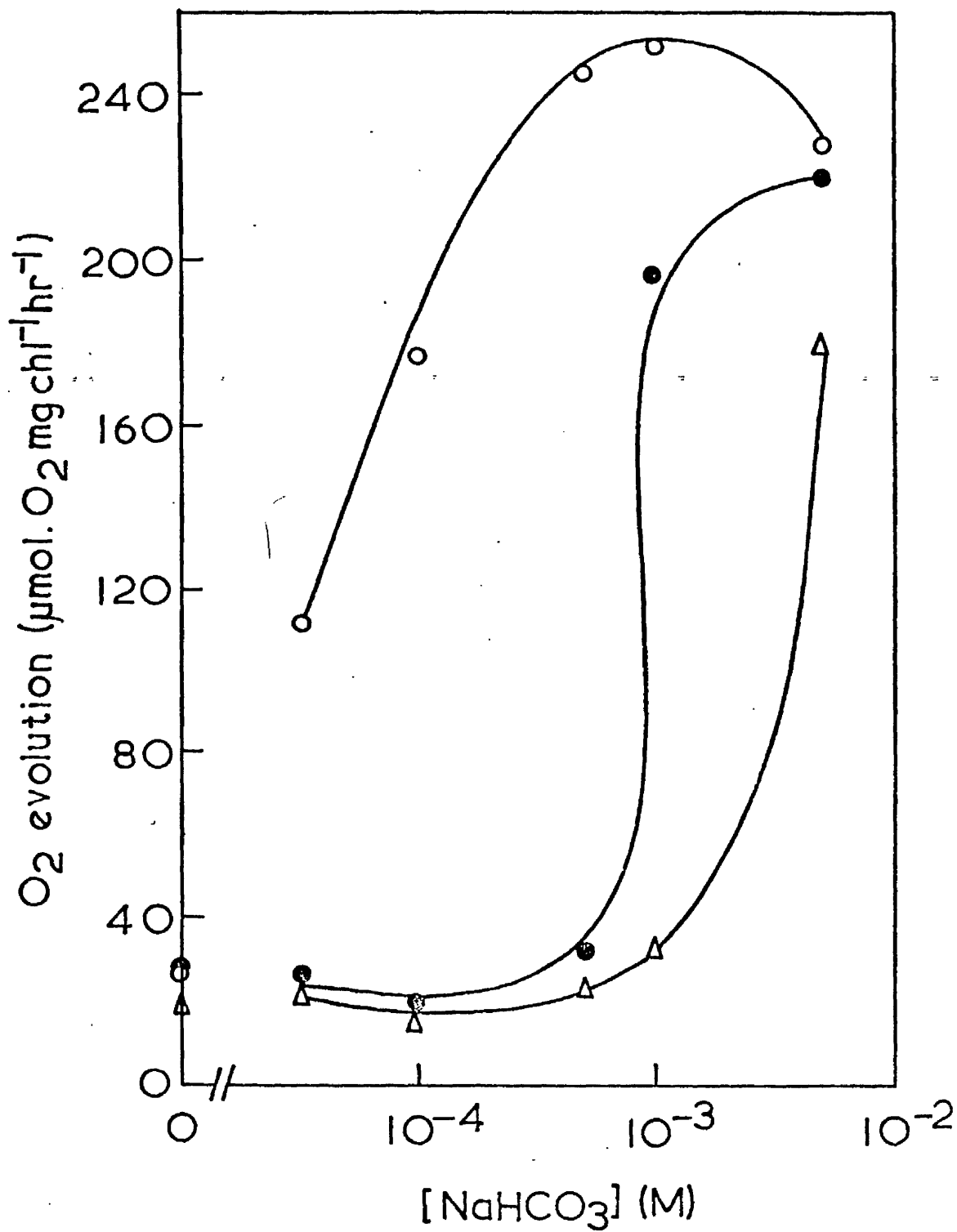


Figure 61. The effect of increasing concentrations of NaHCO₃ on the inhibition of steady state evolution of oxygen in Chlorella by Pb(NO₃)₂. (o), no additions; (●), with the addition of 10⁻³ M Pb(NO₃)₂; (Δ), with the addition of 5.0 x 10⁻³ M Pb(NO₃)₂.

Therefore the effect of adding various concentrations of ZnSO_4 to the algae was investigated on oxygen evolution using CO_2 as the terminal electron acceptor. Figure 62 shows the results obtained under the same conditions as described for the copper experiments. Very little inhibition of oxygen evolution was observed, even at concentrations as high as circa $2 \times 10^{-2}\text{M}$ ZnSO_4 the oxygen evolved was still about 75% of the control value suggesting that unlike copper and mercury, zinc does not directly inhibit photosynthesis at short times. Only at concentrations higher than 10^{-3}M ZnSO_4 was any inhibition noted. This very slight inhibition might be explained either by binding bicarbonate as suggested for the action of lead ions or possibly by slightly altering the pH of the solution, since the pH titration with ZnSO_4 suggested that a slight binding occurs with MOPS buffer. At lower concentration of ZnSO_4 the binding would be insignificant but at the relatively high concentrations of 10^{-2}M Zn^{2+} binding to the buffer might become more significant, preventing the buffer from maintaining the pH. Variations in pH might be expected to cause variations in the rate of photosynthesis in Chlorella (Heldt et al. 1978) and might also effect the concentration of bicarbonate in the medium.

Since no direct inhibition of photosynthesis by ZnSO_4 was observed no further experiments were conducted with this metal.

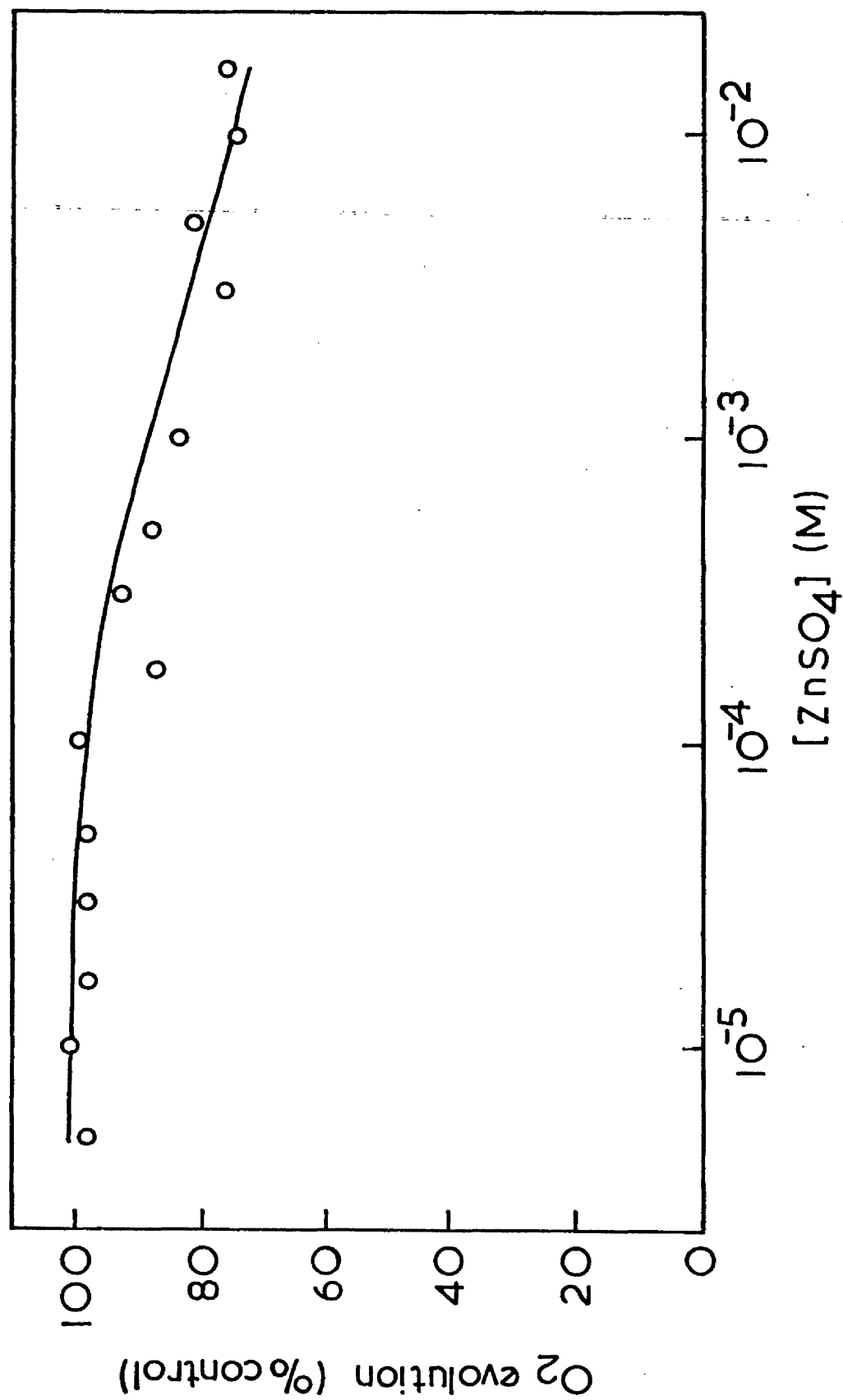


Figure 62. The effect of increasing concentrations of ZnSO_4 on the steady state evolution of oxygen in Chlorella.

The control rate was $137 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{hr}^{-1}$.

CHAPTER 5. CONCLUSIONS

The experiments carried out in this thesis were designed to investigate the light reactions of photosynthesis in the unicellular green alga Chlorella emersonii. Two aspects of this topic were studied in detail; firstly the slow phase of absorption increase at 515nm was studied with an aim to gaining further information on its origin and mechanism. Secondly, the effects of various toxic heavy metal ions were studied to gain a greater understanding of their modes of toxicity. The unicellular alga was used for these studies since it provided an intact, in vivo plant system which was in a form amenable to investigation by a number of techniques, without the necessity for disruptive procedures of isolating chloroplasts, or other subcellular fragments from the original living material. The techniques available during the period of research required a small volume of a suspension of the photosynthetic material and Chlorella have provided an adequate although not perfect source of plant material with these objectives in mind.

However the alga suffered from one major disadvantage which has hampered the studies carried out; the intact cell, with its cell wall, plasmallema and cytoplasm prevents a number of compounds from gaining easy accessibility to the chloroplast and its photosynthetic membranes. In order chemically to isolate various segments of the light reactions it is necessary for compounds such as inhibitors, artificial electron donors and acceptors, redox titration mediators etc to be freely available to the thylakoid membranes where they act in their appropriate manner. This has not always been found to be the case in Chlorella. In the first part of this study (Chapter 3) in vitro photosynthetic preparations were obtained from spinach, pea and photosynthetic bacteria but they were not found to exhibit the particular properties which were necessary for the studies carried out.

Thus notwithstanding these disadvantages various biophysical techniques have been used which allow the light reactions of photosynthesis to be studied without the use of chemical compounds which do not permeate freely into the intact cell. Compounds which, on the other hand, have been found to permeate freely into the alga have provided useful information both on the slow phase of absorption at 515nm and on the modes of toxicity of the heavy metals, copper, mercury, and lead.

It has been found that a number of compounds; CCCP, FCCP, DNP, KCN, HgCl_2 and CuSO_4 all have the property of increasing the apparent size of the slow phase of the field indicating absorption change at 515nm, whilst at the same time markedly decreasing the rate of decay of the absorption change. The classical uncoupling compounds CCCP, FCCP and DNP are required to be at sub-uncoupling concentrations for this effect to occur and since KCN, HgCl_2 and CuSO_4 elicit similar effects it is suggested that these compounds behave as low concentrations of uncouplers in addition to other modes of action which have been reported in the literature and suggested in this thesis. Whether this general behaviour results from a mild uncoupling effect or from secondary effects of the various compounds has not been resolved fully by these studies although various possible mechanisms have been discussed. One possible mechanism by which all the compounds and dark adaption may exert the stimulation of the slow phase revolves around the ability to slow down the decay of the absorption change and thereby make the slow increase more easily observed. Others have used compounds such as tri-N-butyltin-chloride (Bourges Bocquet, 1980) or p-benzoquinone (Diner and Joliot, 1976) to slow down the rate of decay in order that the slow phase of absorption may be observed more easily which concurs with the above mechanism. The nature of the slowing down of the decay rate has also not been resolved but it seems likely that an inhibition of the flow of protons through the coupling factor by all these compounds is the probable mode of action rather than an effect on the electron transport chain itself (Diner and Joliot, 1976).

The experiments carried out here, particularly the deconvolutions of the CCCP, KCN and HgCl_2 data in Chapter 3 tend to agree with the hypothesis. Other modes of stimulating the slow phase must not be ignored and are discussed in the relevant sections.

The slow phase has been found to be associated with the reactions involving PSI by experiments using fluorescence and absorption spectroscopy but attempts to isolate the components of the electron transport chain in the cytochrome region of the intersystem carriers responsible for the slow phase have only been interpreted cautiously due to the lack of confidence in the methods of derivation of the absorption changes thought to represent the redox properties of cytochrome f and cytochrome b₅₆₃. Therefore, definite conclusions supporting or disagreeing with the various models put forward (see section 1.5.3.5.1) for the electron transfer chain involving the slow electrogenic step have not been forthcoming and the true mechanism remains unclear.

In order to derive further information on the slow phase and its stimulation, without the problems of permeability encountered in intact algal cells, similar studies have been carried out using the in vitro isolated chloroplasts, both intact and osmotically shocked. However the stimulation of the slow phase seen in Chlorella was not found in chloroplasts; thus further studies were not possible. The studies with chloroplasts did yield other useful data however; firstly the in vitro preparations were found to require different light pretreatments to the algae for maximal appearance of the slow phase and secondly it was found that the presence of divalent cations was required for the retention of the components necessary for the appearance of the slow phase. This suggested different steady state redox poising in the algae and chloroplasts and further indicated that the slow phase required a loosely bound component for its observation.

It is unfortunate that the two systems proved non-exchangeable with regards to the stimulatory effects mentioned since both systems would appear to be required for their various advantages over the other system when studying photosynthetic activities.

The studies on the heavy metal toxicities proved slightly more successful and a number of inhibitory activities were shown for copper and mercury though not so for lead and zinc. Both copper and mercury, in addition to the effect discussed above, were found to be particularly inhibitory towards the PSII site of the light reactions. This is in agreement with the literature as PSII has been reported to be the most common site affected by heavy metals (see section 1.6). The techniques available were generally those used for monitoring PSII activities and so other sites of inhibition at the PSI and intersystem carrier sites cannot be ruled out as further possible sites of copper and mercury toxicity, particularly since both metals were found to decrease the extent of the 515 absorption change attributed to PSI. The experiments have shown conclusively that both these metals act quickly, within five minutes of their incubation with the algae. Therefore it has been shown that in addition to the slower toxic effects of these metals reported on cell division and growth (see section 1.6) high concentrations act directly on the photosynthetic apparatus. Thus pollution by these metals may be directly observed by decreased rates of photosynthetic activity. The experiments also suggested that heavy metals are bound easily by buffers, bicarbonate and other soluble ions forming insoluble complexes. Therefore it is suggested that the toxicity of heavy metals will vary depending on the presence of other compounds in the environment which form insoluble complexes with the metals and will tend to decrease their availability to plants and other living systems by chemical binding.

Environments containing these compounds such as areas containing hard water would be less susceptible to heavy metal pollution than those with lower concentrations of the ions such as those areas containing soft water. Indeed, the studies on lead showed that its primary inhibitory effect was to bind with the bicarbonate ions preventing their availability for photosynthesis. It is therefore suggested that the toxicity of heavy metals will be dependent on a large number of external factors such as cell number, chemical availability of the metal ions, as well as the particular species of organisms present since lead and zinc did not directly affect photosynthesis here in Chlorella emersonii but are well-known to inhibit higher plant chloroplasts and other algae (see sections 1.6.3 and 1.6.4).

A factor not investigated but which in hindsight might have yielded further information in both sections and lessened the variability between different cultures of algae is that of the cycle of cell growth. Although each culture of algae was harvested after similar periods of time, it is probable that the samples contained cells of differing developmental stages since no light/dark photoperiod was used during growth. The use of synchronous growth cultures would probably have limited the variability between different cultures and furthermore may have shown that the effects observed both with heavy metals and in the slow phase studies depended on the state of division or development of the algae. This might have been particularly relevant for the heavy metal studies since copper has been reported to inhibit cell division in Chlorella and hence to inhibit the liberation of autospores, which leads to an accumulation of photosynthetic products (Steeman-Nielsen et al. 1969). This might have been more thoroughly investigated had all the cells been at the same development stage.

Furthermore Senger (1977) has shown that the sensitivity of various unicellular green algae to photosynthetic inhibitors such as DCMU, DBMIB and Simazine depends markedly on the developmental stage of the life cycle of the algae. He suggests that this may be a consequence of changes in the permeability of the cell membranes during the life cycles. A similar phenomena has also been reported for the compound dinitrophenol (DNP) by Ried et al. (1962).

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APPENDIX 1

Composition of Culture Solutions, Nutrient Media and Isolation Media

(a) Culture Solution for Chlorella (Barber, 1968)

The culture medium was normally made up in 2 litre batches and contained the following nutrients;

10 mls of 1M KNO_3
1.0 mls of 1M K_2HPO_4
1.0 mls of 1M KH_2PO_4
4.0 mls of 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
0.5 mls of 1M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
4.0 mls of Hutner's Trace Elements (see below)

made up to 2 litres with distilled H_2O and autoclaved at 8 lbs in^{-2} for 10 minutes in Dreschel bottles which were plugged with cotton wool and sealed with tin foil to prevent re-contamination. The media were stored in a cold room.

Hutner's Trace Elements

25g of EDTA (Disodium salt)
11g of ZnSO_4
5.7g of H_3PO_4
2.2g of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$
2.5g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
0.84g of $\text{Co}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$
0.78g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
0.55g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$

made up in 500 mls distilled H_2O .

APPENDIX 1 (Cont.)

(b) Nutrient Solution for Spinach and Pea Growth (Baker et al. 1978)

The nutrient solution was normally made up in batches of 40 litres containing the following:

250 mls of 1M KNO_3

160 mls of 1M $\text{Ca}(\text{NO}_3)_2$

80 mls of 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

40 mls of 1M KH_2PO_4

160 mls of 1M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

25 mls of the trace element solution (see below)

made up in 40 litres of distilled H_2O .

Trace Elements

0.425g of H_3BO_4

0.284g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$

0.0348g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$

0.0124g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

0.03856g of Na $\text{MoO}_4 \cdot 2\text{H}_2\text{O}$

1.40959g of NaFe EDTA

made up in 100 mls distilled H_2O .

APPENDIX 1 (Cont.)

(c) Culture Solution for the Photosynthetic Bacteria (Bose, 1963)

Carbon-succinate medium

20 mls of conc. base (see below)
 20 mls of KH_2PO_4 and K_2HPO_4 (1:1 molar solutions)
 5 mls of NH_4SO_4 (10% w/v)
 10 mls of Potassium succinate (see below)
 1 ml of Growth factors (see below)
 1 g of Casamino acids

made up in 1 litre of distilled H_2O and autoclaved at 15 lb in⁻² for 15 minutes.

Conc Base

10.0 g of Nitroacetic acid, pH 6.8 with 5N KOH
 14.45 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 3.4 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$
 4.25 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$
 99.0 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
 50.0 mg of Nicotinic acid
 25.0 mg of Aneurine HCl
 0.5 mg of Biotin
 50 ml of Metos 44 (see below)

made up in 1 litre of distilled H_2O .

APPENDIX 1 (Cont.)

Potassium Succinate

To solid succinic acid (for 1 M) add KOH until pH 6.8, then add H₂O.

Growth Factors

2 mg Biotin
50 mg NaHCO₃
100 mg Nicotinic acid
50 mg Aneurine HCl
100 mg Amino benzoic acid

made up in 100 mls H₂O and boiled to dissolve.

Metos 44

0.5 g EDTA
2.10 g ZnSO₄·7H₂O
1.0 g FeSO₄·7H₂O
3.08 mg MnSO₄·4H₂O
78.4 mg CuSO₄·5H₂O
49.6 mg CoNO₃·6H₂O
35.4 mg disodium tetra borate: 10 H₂O

made up in 100 mls distilled H₂O and 2 drops of H₂SO₄.

APPENDIX 1 (Cont.)(d) Grinding Medium for Chloroplast Isolation (Stokes and Walker, 1971)

0.33 M Sorbitol
50 mM Na_2HPO_4
50 mM KH_2PO_4
5 mM MgCl_2
0.1 % NaCl
0.2 % Sodium isoascorbate

adjusted to pH 6.5 with HCl.

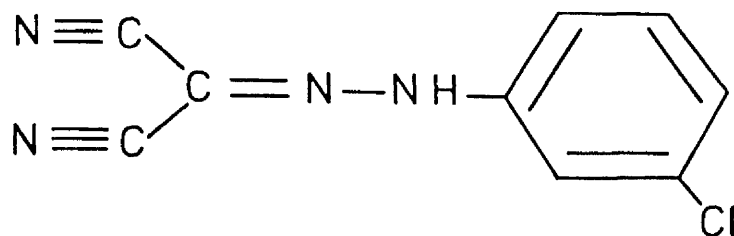
(e) Assay Medium for Chloroplasts (Stokes and Walker, 1971)

0.33 M Sorbitol
2 mM EDTA
1 mM MgCl_2
1 mM MnCl_2
50 mM HEPES buffer

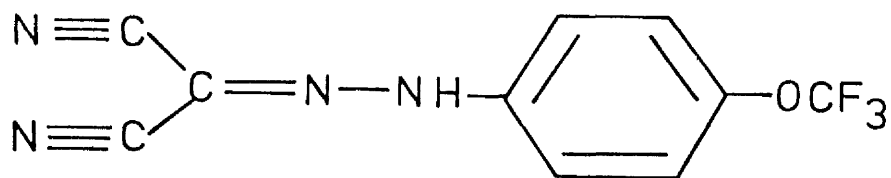
adjusted to pH 7.6 with KOH.

Structures of three uncouplers used in experiments; CCCP - Carbonyl cyanide-m-chlorophenyl hydrazone, FCCP - Carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone, DNP - 2,4 - dinitrophenol.

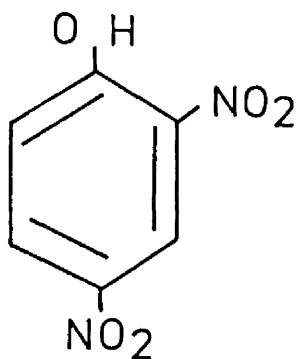
CCCP



FCCP



DNP



APPENDIX 3

Derivation of ϕ_{pmax} from F_V/F_m where $F_V = F_m - F_O$.

$$\phi_{F_O} = \frac{k_f}{k_f + k_h + k_t + k_p}$$

$$\phi_{F_m} = \frac{k_f}{k_f + k_h + k_t}$$

$$\frac{F_m - F_O}{F_m} = \frac{F_V}{F_m} = \frac{\left(\frac{k_f}{k_f + k_h + k_t} \right) - \left(\frac{k_f}{k_f + k_h + k_t + k_p} \right)}{\left(\frac{k_f}{k_f + k_h + k_t} \right)}$$

$$= \frac{k_f}{k_f + k_h + k_t} - \frac{k_f}{k_f + k_h + k_t + k_p} \times \frac{k_f + k_h + k_t}{k_f}$$

$$= \frac{k_f(k_f + k_h + k_t + k_p) - k_f(k_f + k_h + k_t)}{(k_f + k_h + k_t) \times (k_f + k_h + k_t + k_p)} \times \frac{k_f + k_h + k_t}{k_f}$$

$$= \frac{(k_f)^2 + k_f k_h + k_f k_t + k_f k_p - (k_f)^2 - k_f k_h - k_f k_t}{k_f(k_f + k_h + k_t + k_p)}$$

$$= \frac{k_p}{k_f + k_h + k_t + k_p} = \phi_{pmax} = \frac{F_V}{F_m}$$

Derivation of Metal Binding Constants for MOPS buffer from the
Displacement of the pH Titration Curve in the Presence of the Metal
(taken from Good et al. 1966).

If it is assumed that the metal forms a co-ordinate bond with the amine nitrogen of the buffer and in so doing competes with protons the following equations express the equilibrium condition.

$$[A] = [N] + [NH^+] + [NM^+] \quad (1)$$

where $[A]$ is the total buffer concentration, $[N]$ is the concentration of free amine, and $[NM^+]$ is the concentration of metal buffer complex and

$$[M] = [M^{2+}] + [NM^+] \quad (2)$$

where $[M]$ is the total metal concentration and $[M^{2+}]$ is the concentration of free metal ion. Each of the two competing processes has its own equilibrium equation. Thus

$$K_a = \frac{[N][H^+]}{[NH^+]} \quad (3)$$

where K_a is the acid dissociation constant of the buffer and

$$K_m = \frac{[NM^+]}{[N][M^{2+}]} M^{-1} \quad (4)$$

where K_m is the metal-buffer binding constant. By substituting equations 1, 2, and 3 in equation 4 the following expression is obtained

$$K_m = \frac{[A] - K_a [NH^+] / [H^+] - [NH^+]}{(K_a [NH^+] / [H^+]) ([M] - [A] + K_a [NH^+] / [H^+] + [NH^+])}$$

It is assumed that all of the buffer is in the protonated form at the beginning of the titration and therefore it is reasonable to assume that half is in the protonated form at the midpoint of the titration curve, or $[NH^+] = [A]/2$. The three values $[H^+]$, K_a , and $[A]$ are all provided by the titration curves observed in the presence and absence of the metal.

APPENDIX 4 (Cont.)

[A] is the concentration of MOPS buffer, 0.01M; [H⁺] from the midpoint pH value in the presence of the metal where $\text{pH} = -\log [\text{H}^+]$; and K_a from the midpoint pH value in the absence of the metal where $\text{pK}_a = -\log K_a$. [M] is the concentration of the metal added before each titration.

APPENDIX 5

Derivation of the expression of the yield of trapping at the reaction centre $\phi_T [1 - \phi_d]$ allowing for radiation less de-excitation of excitons at the reaction centre (adapted from Butler and Kitajima, 1975)

$$\phi_f = \frac{k_f}{k_f + k_h + k_t + k_T}$$

where $k_T = k_T [A + (1 - A)\phi_d]$

k_T = rate constant for trapping by the reaction centre of exciton energy.

A = the fraction of open reaction centres.

ϕ_d = the quantum yield of radiation less de-excitation at the closed reaction centre.

$$\phi_{F0} = \frac{k_f}{k_f + k_h + k_t + k_T [1 + (0 \times \phi_d)]}$$

$$\phi_{Fm} = \frac{k_f}{k_f + k_h + k_t + k_T [0 + (1 \times \phi_d)]}$$

$$\begin{aligned} \frac{\phi_{Fm} - \phi_{F0}}{\phi_{Fm}} &= \frac{F_v}{F_m} = \frac{\left(\frac{k_f}{k_f + k_h + k_t + k_T [\phi_d]} \right) - \left(\frac{k_f}{k_f + k_h + k_t + k_T [1]} \right)}{\frac{k_f}{k_f + k_h + k_t + k_T [\phi_d]}} \\ &= \frac{k_f}{k_f + k_h + k_t + k_T [\phi_d]} - \frac{k_f}{k_f + k_h + k_t + k_T [1]} \times \frac{k_f + k_h + k_t + k_T [\phi_d]}{k_f} \\ &= \frac{k_f(k_f + k_h + k_t + k_T [1]) - k_f(k_f + k_h + k_t + k_T [\phi_d]) \times k_f + k_h + k_t + k_T [\phi_d]}{(k_f + k_h + k_t + k_T [\phi_d]) \times (k_f + k_h + k_t + k_T [1]) \times k_f} \\ &= \frac{(k_f)^2 + k_f k_h + k_f k_t + k_f k_T [1] - (k_f)^2 - k_f k_f - k_f k_h - k_f k_T [\phi_d]}{k_f(k_f + k_h + k_t + k_T [1])} \\ &= \frac{k_T [1] - k_T [\phi_d]}{k_f + k_h + k_t + k_T [1]} \\ &= \frac{k_T [1 - \phi_d]}{k_f + k_h + k_t + k_T [1]} = \phi_T [1 - \phi_d] = \frac{F_v}{F_m} \end{aligned}$$

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ERRATA

In the legends to figures 20, 22, 23, 32, 34, 39, 40, 41, 45, 46, 53, 59 and tables 7 and 9, for "the effect of on the fast phase, slow phase", read "the effect of on the extent of the fast phase, the extent of the slow phase".